Subhra K. Biswas · Alberto Mantovani
Editors

Macrophages: Biology and Role in the Pathology of Diseases

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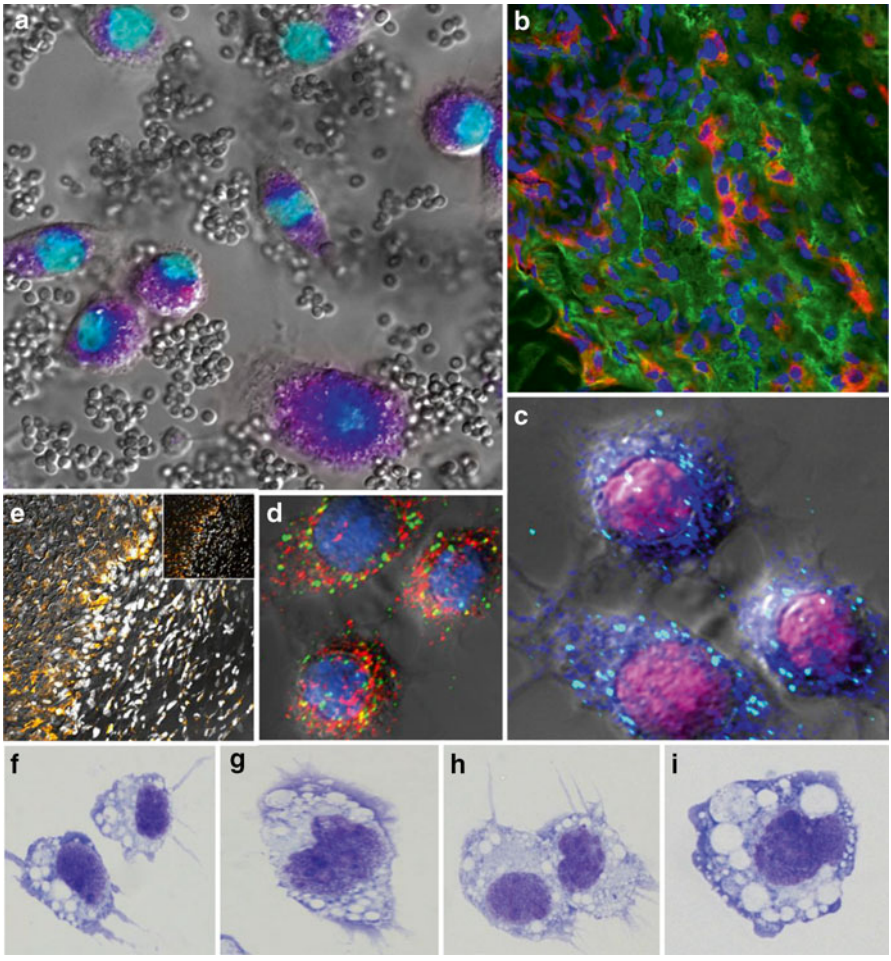

Macrophage interaction in different contexts and tissue distribution.
a Macrophages challenged with *Aspergillus fumigatus* conidia; **b** Macrophage (red) interaction with matrix (green); **c-d** Internalization of West Nile Virus dsRNA (cyan/green) by macrophages; **e** Tumor-stroma interaction showing tumor associated macrophages (yellow) in pancreatic ductal carcinoma. **a-e** Courtesy: Andrea Doni (Istituto Clinico Humanitas, Rozzano, Italy); **f** Yolk sac macrophage; **g** Lung macrophage; **h** Brain macrophage (Microglia); **i** Splenic macrophage. **f-i** Courtesy: Florent Ginhoux and Guillaume Hoeffel (SIgN, A*STAR, Singapore)

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Macrophages: Biology and Role in the Pathology of Diseases

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Preface

Macrophages are a key component of the innate immune system and play an essential role in host defense and homeostasis. On the one hand, these cells contribute to host defense by triggering inflammation, displaying microbicidal and tumoricidal properties, regulating the activation of adaptive immunity, resolving inflammation, and promoting tissue repair. On the other hand, they perform essential trophic functions contributing to developmental processes in many organs or tissues like the brain, mammary gland, and bones. Thus, macrophages are extremely versatile cells that can efficiently respond to the tissue microenvironmental cues by polarizing to distinct functional phenotypes depending on the functions they need to perform. Indeed, functional plasticity and diversity are hallmarks of these cells.

Macrophages may also play a detrimental role. An overwhelming body of literature supports a crucial role for these cells in pathogenesis. The list is exhaustive, including cancer, metabolic syndrome, sepsis, allergy, immunodeficiency, autoimmune disease, etc.—impacting virtually every major disease that we know. These observations in turn suggest macrophages and their related molecules as potential targets for therapeutic applications.

In view of the above, macrophages have emerged as key players in homeostasis, host defense, and disease. However, in the last 10 years, a phenomenal amount of research has shed new light on our understanding of these cells including:

- New concepts on the origin of macrophages
- The trophic functions of macrophages and their contribution to homeostasis
- Emergence of new myelomonocytic subsets and their relationship to macrophages
- New tools to study monocytes and macrophages in vivo (such as fate mapping and novel transgenic models)
- The concept of macrophage polarization and definition of distinct polarization states
- Systems biology of macrophages: characterization of the transcriptional and post-transcriptional networks that regulate macrophage polarization and function

- Profiling of macrophages in vivo in various disease conditions (e.g., tumors, metabolic syndrome, resolution of inflammation, parasite infection, etc.) and a better understanding of their contribution in disease progression.

These developments called for a reevaluation and update of our understanding of macrophages. Thus, we hope that this book is both timely and topical in presenting a state-of-the-art understanding of these cells in health and disease.

We sincerely thank all the authors who have kindly contributed to this endeavor. We would also like to thank Greg Baer and the Springer staff who were involved in coordinating the production of this book.

Singapore Subhra K. Biswas, PhD
Milan, Italy Alberto Mantovani, MD

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Part I
Macrophage: Origin, Activation and Polarization

Chapter 1
Blood Monocytes and Their Subsets in Health and Disease

Loems Ziegler-Heitbrock

1.1 Origin and Fate of Blood Monocytes

Monocytes are white blood cells, which are slightly larger than lymphocytes, and are characterized by an irregular nucleus and a light blue cytoplasm. Monocytes develop in bone marrow from progenitor cells. Here the common myeloid precursor gives rise to the granulocyte–monocyte precursor eventually leading to development of monocytes. From the bone marrow monocytes go into blood and then migrate into various tissues, where they develop into macrophages. This simple concept by-and-large holds true but there are some additional routes of monocyte travel in that, for instance, it has been demonstrated in the mouse that blood monocytes can return to the bone marrow (Varol et al. 2007) and that the spleen forms a reservoir, from which monocytes can be mobilized (Swirski et al. 2009). Also, it is still unclear to what extent monocytes under homeostatic conditions replenish the macrophage populations in the various tissues. Here evidence in the mouse suggests that monocytes do not contribute to the microglia of the brain (Ginhoux et al. 2010) while they can replenish macrophages in tissues like kidney and lung (Schulz et al. 2012). Also, bone marrow transplantation experiments show that lung macrophages will become of donor type, demonstrating that they are derived from immigrating monocytes (Thomas et al. 1976; Hashimoto et al. 2013). In addition, monocytes can differentiate into dendritic cells (DC) but we do not know as to whether monocytes give rise to bona fide tissue DCs or whether most tissue DCs rather derive from specific DC precursor cells, which are distinct from monocytes. Whatever their fate,

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it had been shown early on that monocytes under homeostatic conditions circulate in blood for 1–3 days (van Furth and Cohn 1968; Whitelaw 1972). Under conditions of infection and inflammation of the tissue chemokines are released locally. These then activate blood monocytes and attract them to this site. It is in these tissues where the monocytes—now termed macrophages—are crucial to innate immune defense and where they are involved in tissue remodelling and repair. Again, mouse studies show that during nematode infection lung macrophages may increase by local proliferation without contribution of blood monocyte influx (Jenkins et al. 2011). But as shown in another inflammation model (peritonitis) immigrating monocytes do contribute to the local population and they can even give rise to proliferating macrophages (Davies et al. 2013). Since the proliferation potential of human macrophages is very limited (Pforte et al. 1993), it remains to be shown whether the concept will apply to man.

In addition to being the progenitors for tissue macrophages the blood monocytes themselves may contribute to defense against infection. When, for instance, microbes enter the blood then monocytes can phagocytose and destroy them.

However, a main reason for studying blood monocytes is that these cells offer a window that provides a view on the state of the monocyte–macrophage system as a whole.

1.2 Monocyte Subsets

Because of the easy access to human blood, evidence for heterogeneity of these cells was first provided in man (Yasaka et al. 1981; Zembala et al. 1984). With the advent of flow cytometry monocyte subsets were then clearly defined in that CD16-positive and CD16-negative monocytes were distinguished (Passlick et al. 1989). Later work dissected the CD16-positive monocytes further into functionally distinct subpopulations (Grage-Griebenow et al. 2001). Together with many additional studies these findings eventually led to a nomenclature for human blood monocytes (Ziegler-Heitbrock et al. 2010), which distinguishes the classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and the non-classical ($CD14^{+}CD16^{++}$) monocytes (see Fig. 1.1)

There is some circumstantial evidence that in man the classical monocytes give rise to intermediate and then to non-classical monocytes and this is based on time course studies in acute infection and after depletion of monocytes with chemotherapy (Ziegler-Heitbrock and Hofer 2013; Dayyani et al. 2004).

Monocyte subsets in man have been studied extensively since the 1980s of the twentieth century (Passlick et al. 1989). By contrast, blood monocyte subsets in the mouse model have gained wider interest only in the twenty-first century (Palframan et al. 2001). In this species similar subsets can be defined using markers Ly6C and CD43, and here the classical monocytes are $Ly6C^{++}$ $CD43^{+}$, the non-classical monocytes are $Ly6C^{+}$ $CD43^{++}$ while the intermediate monocytes have intermediate levels for both markers (see Fig. 1.2).

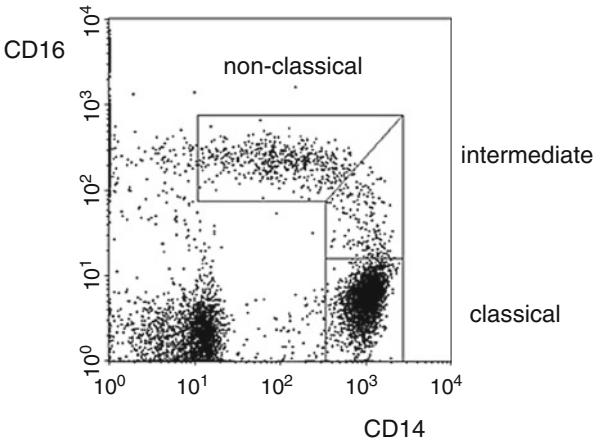

Fig. 1.1 The three human monocyte subpopulations. *Dot plot* kindly provided by Dr. Thomas PJ Hofer, Helmholtz-Zentrum Muenchen, Gauting, Germany

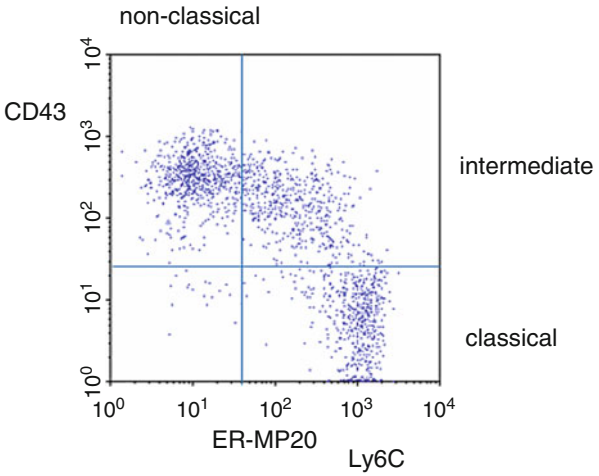

Fig. 1.2 The three mouse monocyte subpopulations. *Dot plot* kindly provided by Dr. Pieter JM Leenen, Erasmus Medical Center, Rotterdam, The Netherlands

The mouse intermediate cells were shown to have monocyte morphology and to exhibit intermediate levels of CD62L, CCR2, and CX3CR1 (Sunderkotter et al. 2004; Santiago-Raber et al. 2011). Using depletion and tracking studies it has been shown clearly in the mouse that classical monocytes give rise to non-classical monocytes (Sunderkotter et al. 2004; Yona et al. 2013). This is also supported by time course studies, which show after injection of a TLR8 or 9 ligand the transient appearance of intermediate monocytes followed by an increase of non-classical monocytes (Santiago-Raber et al. 2011; Kwissa et al. 2012). For the mouse model the half-life of the classical and non-classical monocytes has been determined (Yona et al. 2013) and was shown to be about 1 day for the classical and about 2 days for the non-classical monocytes. Similar subset specific data for man are outstanding.

Comparison of monocyte subsets between man and mouse has shown many similarities but also many important differences between these species (Ingersoll et al. 2010). Therefore findings and concepts based on monocytes in the mouse, be it under physiological conditions or in disease models, cannot be transferred to man directly, but they need to be tested and confirmed in man. This conclusion is supported by a recent gene expression study of three relevant inflammatory conditions (burn, trauma and LPS infusion mimicking sepsis), which demonstrated that the mouse model does not recapitulate any of the human conditions (Seok et al. 2013).

The existence of monocyte subpopulations is evident in man and mouse but also in many other species like non-human primates (Munn et al. 1990; Kim et al. 2010a), rats (Ahuja et al. 1995; Scriba et al. 1997; Yrlid et al. 2006), and pigs (Sanchez et al. 1999; Fairbairn et al. 2011). Therefore, it is clear that monocytes in man, mouse, and all the other species cannot any longer be addressed as one population. What is required for state-of-the-art studies is a clear definition of the subset that is being analyzed.

1.3 Cell Lines as Models

The human cell lines of the monocyte lineage, which are most widely used, are U937, THP1, and Mono Mac 6. U937 (Sundstrom and Nilsson 1976) was the first cell line of this lineage to be described and represents a fairly immature cell type with no or little expression of the monocyte marker CD14. THP-1 (Tsuchiya et al. 1980) is somewhat more mature and can be further differentiated with Vitamin D3. Mono Mac 6 (Ziegler-Heitbrock et al. 1988) expresses CD14 and responds rapidly to lipopolysaccharide. Also for Mono Mac 6 treatment with VD3 will lead to further maturation and treatment with TGFbeta plus VD3 has been used in a series of studies on leukotrienes (Werz et al. 1997, 2000). These cell lines have proven useful models for molecular studies since gene transfer is more efficient compared to primary blood monocytes. However, none of these cell lines expresses CD16 and therefore they cannot serve as a model for non-classical or intermediate monocytes.

In the mouse several cell lines representing monocytes and macrophages are available. Here the WEHI-3B (Warner et al. 1969) expresses the Ly6C antigen but the cells are non-phagocytic and therefore may represent bone marrow monocytes (Leenen et al. 1986, 1990).

While there may be no cell line representing the Ly6C^{++} classical blood monocyte, the Pu5 line (Ralph et al. 1974) appears to be similar to the non-classical blood monocyte.

The cell lines J774 (Ralph et al. 1975), P388D1 (Koren et al. 1975), and RAW 264.7 (Raschke et al. 1978) are more mature beyond the level of the blood monocyte. They are representative of tissue macrophages, specifically they may represent newly emigrated exudate macrophages. The availability of several cell lines that represent macrophages in the mouse but not in man may indicate that the normal, non-transformed mature tissue macrophages have more proliferative potential in these animals compared to man.

1.4 Monocyte Function

1.4.1 General Remarks

Blood monocytes are extremely sensitive cells and any manipulation can alter their properties. The typical ficoll-hypaque isolation technique for mononuclear cells can already activate the monocytes and influence receptor expression (Lundahl et al. 1995). Also positive selection of monocytes with antibodies against cells surface molecules can lead to activation since the antibody binding may trigger signaling pathways once the cells are put in culture. Finally, we have to be aware of the high sensitivity of monocytes to stimulation by LPS and other microbial products. These microbial products can inadvertently contaminate materials and reagents that are brought in contact with monocytes during purification and in culture. Pre-activation of monocytes by all of these mechanisms may lead to enhanced or decreased responses of the monocytes in subsequent functional studies. Therefore the favored strategy for the study of monocytes involves minimal processing and no-touch isolation. The preferred approach is the whole blood analysis.

1.4.2 Phagocytosis

Monocytes belong to the professional phagocytes and they are able to engulf huge particles. The phagocytosis can proceed via the conventional process but there are specific processes like coiling phagocytosis used for uptake of Borrellia and Legionella (Horwitz 1984) or looping phagocytosis (Clemens et al. 2012). There is a multitude of receptors involved in phagocytosis like receptors for the Fc-portion of immunoglobulin, complement receptors, scavenger receptors, and lectins (Underhill and Ozinsky 2002). Also there is phagocytosis of apoptotic cells, which is important to development and repair (Lauber et al. 2003).

While phagocytosis of apoptotic cells is a silent process, phagocytosis of microbes will go along with cytokine production and antigen presentation. These processes are crucial tasks of tissue macrophages but blood monocytes can also execute all of these steps and thereby may contribute to immune defense against infection.

When looking at monocyte subsets it has been noted that the CD16-positive monocytes show a Fc-receptor-phagocytosis signature (Zhao et al. 2009). However, phagocytosis of antibody coated erythrocytes was found decreased in the CD16-positive monocytes (Passlick et al. 1989). Also, uptake of *Escherichia coli* bacteria was lower (Dayyani et al. 2004). On the other hand higher phagocytic activity in the CD16-positive monocytes was reported for 0.5 μ latex beads (Mosig et al. 2009) and for *E. coli* (Nockher and Scherberich 1998). These conflicting results may be due to different handling of the cells in the different studies or to different properties of the various particles used. A comprehensive study looking at the entire spectrum of

particles, decorated with the various ligands, is still outstanding for the different monocytes subsets. Also, looping and coiling phagocytosis have not been looked at in classical, intermediate, and non-classical monocytes. However, when it comes to uptake of apoptotic granulocytes, highest activity was reported for both intermediate and non-classical monocytes, while classical monocytes showed low activity (Mikolajczyk et al. 2009).

1.4.3 Cytokine Production

Human blood monocytes have been used extensively to study production of a whole array of cytokines and this includes the major pro- and anti-inflammatory cytokines TNF and IL-10, respectively.

Looking at monocyte subsets the CD16-positive cells were shown to produce more TNF in response to LPS (Belge et al. 2002; Kanai et al. 2007; Mikolajczyk et al. 2009; Dimitrov et al. 2013; Balboa et al. 2013; Hearps et al. 2012a) and to TLR7/8 ligands (Cros et al. 2010). Higher TNF production by non-classical monocytes in man was also reported for cells stimulated with tumor microvesicles (Baj-Krzyworzeka et al. 2010).

The higher cytokine production in the CD16 positive cells is not seen in all reports, which may be due to variability among the blood donors or alternatively to differential effects of cell processing before the assay. Most convincing are therefore results without manipulation, which are obtained using whole blood assays (Belge et al. 2002).

Also, chemokines CCL5 and CXCL10 were found highest in the CD16-positive monocytes in sepsis patients and when control donor monocytes were activated with lipid A in vitro. This higher expression was shown to be supported by signaling mediated by CD16 (Shalova et al. 2012).

Work by the Pryjma-laboratory has shown that among the CD16-positive monocytes the intermediate monocytes can show very high TNF production (Skrzeczynska et al. 2002; Mikolajczyk et al. 2009). Others also found TNF levels in intermediate monocytes to be highest among the monocyte subsets (Dimitrov et al. 2013).

Also, for IL-10 highest levels were shown for the intermediate monocytes (Skrzeczynska et al. 2002; Mikolajczyk et al. 2009).

In the mouse whole blood analysis of LPS stimulated cells has demonstrated higher levels of TNF in the non-classical monocytes (Burke et al. 2008). Furthermore, studies by Auffray et al. have shown that non-classical monocytes after emigrating into the peritoneum during infection with L. monocytogenes produce much higher levels of TNF than classical monocytes (Auffray et al. 2007).

Taken together monocytes can produce a broad array of cytokines and chemokines. The differential expression of these mediators among monocyte subsets can be explained by differential expression of cells surface receptors and intracellular signaling molecules.

1.4.4 Antigen Presentation

Presentation of exogenous peptide antigen to T helper cells is a crucial step in the adaptive immune response. In this process exogenous protein is taken up, digested, and loaded into MHC class II molecules, which are then shuttled to the cell surface of the antigen-presenting cell. Here the Class II-peptide complex is recognized by specific T cell receptor molecules on the T helper cells. The interaction is stabilized by the CD4 receptor and this results in T cell activation, proliferation, and production of cytokines. Monocytes in man are among the few cell types, which constitutively express high levels of MHC class II and these cells therefore can support T cell activation driven by antigen or superantigen. When it comes to MHC class II cell surface expression among monocyte subsets it was reported early on that the CD16-positive monocytes show a higher level for HLA-DR (Passlick et al. 1989; Grage-Griebenow et al. 2001). Also the CD16-positive cells were shown to induce higher levels of IFN-gamma in T cells when stimulated with mycobacterial antigen (Grage-Griebenow et al. 2001). More recently superantigen-driven T cell proliferation was found to be strongest for the intermediate monocytes (Zawada et al. 2011).

1.4.5 Migration

Migration of leukocytes is governed by chemokines, which target g-protein coupled 7-transmembrane chemokine receptors found on the cell surface (Murphy et al. 2000). The process of emigration of leukocytes from blood into tissue can be separated into rolling, activation, arrest, and transmigration and this has been covered in many reviews on the various leukocytes including monocytes (Ley et al. 2007).

Monocytes express predominantly receptors of the CCR type and respond to the respective ligands but there also is a role for CXC receptor ligand pairs. CX3CR1 is strongly expressed by monocytes; this chemokine receptor is, however, more closely related to the CCR receptors, since it is encoded in the major CCR cluster on the human chromosome 3 (Combadiere et al. 1995). Among monocyte subsets there is a lack of CCR2 expression by the non-classical monocytes both in man and in the mouse (Weber et al. 2000). Accordingly, classical monocytes respond exclusively to CCL2, which is a chemokine induced under many inflammatory and infectious conditions. Also under homeostatic conditions CCR2 is required for exit of classical monocytes from bone marrow into blood, such that the $Ly6C^{++}$ monocytes are lower in blood of $CCR2^{-/-}$ mice. Furthermore, during infection there is less of an increase of classical monocytes in the $CCR2^{-/-}$ animals (Serbina and Pamer 2006). For CX3CR1 the expression levels were shown to be higher on the non-classical monocytes at both the mRNA and the protein level (Ancuta et al. 2003). This differential expression goes along with a preferential arrest and migration of the CD16-positive monocytes when triggered by fraktalkine, i.e. the CX3CR1-ligand.

Studies in probands with excessive exercise have shown a selective increase in the CD16-positive monocytes within minutes (Steppich et al. 2000; Dimitrov et al. 2013), an effect attributed to the action of catecholamines. In fact, Dimitrov et al. (2010) could show that infusion of epinephrine will increase the number of CD16-positive monocytes. The rapid mobilization is conceivable when we assume that the non-classical monocytes reside in the marginal pool, i.e., they localize to vascular endothelium (Steppich et al. 2000). This concept is supported by work in the mouse, in which slow movement of non-classical monocytes on vascular endothelium was demonstrated in vivo (Auffray et al. 2007). This puts the non-classical monocytes in the pole position such that in case of an inflammatory signal they can rapidly emigrate into tissue.

1.4.6 Maturation to Macrophages and DC

When monocytes go into tissue then they mature into quite diverse types of macrophages. These show different phenotypic and functional properties that depend on the type of tissue. Pathophysiological processes like malignancy and different inflammatory processes, which are characterized by different cytokine milieus, will also shape the macrophage properties.

To what extent the immigrating monocytes contribute to the pool of macrophages may vary with the type of tissue. For nematode infection it has been shown in the mouse that local proliferation rather than influx of monocytes replenishes lung macrophages (Jenkins et al. 2011).

Many studies have tried to recapitulate the process of monocyte-to-macrophage differentiation in vitro in that monocytes are put into culture without or with addition of different cytokines. In such a system monocytes can develop into large macrophages within a few days (see Fig. 1.3).

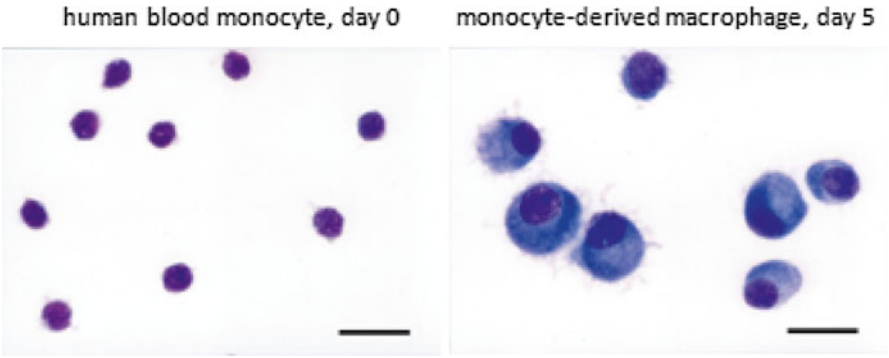

Fig. 1.3 Morphology of monocytes and 5 day monocyte-derived-macrophages. $CD14^{++}CD16^{-}$ classical blood monocytes were isolated by MACS and cultured for 5 days in low attachment plates in the presence of M-CSF at 1,000 ng/mL. Cytospin of monocytes and MDM kindly provided by Dr. Thomas PJ Hofer and Kerstin Skokann, Helmholtz-Zentrum Muenchen, Gauting, Germany

Here culture in the presence of IFNgamma plus LPS generates classically activated macrophages with pro-inflammatory features (M1) while culture in the presence of IL-4 or IL-13 will generate alternatively activated macrophages (M2) (Gordon 2003).

Looking at different monocyte subsets Frankenberger et al., using no-touch selection techniques, have shown a differential expression of cell surface molecules and a higher phagocytosis capacity by macrophages derived from CD16-positive monocytes as compared to macrophages derived from classical monocytes (Frankenberger et al. 2012). Earlier it had been shown that macrophages generated in co-cultures of T cells with CD16-positive monocytes produced much higher levels of CCL2 and CCL24 than similar cultures with CD16-negative monocytes (Ancuta et al. 2006). While no further characterization of the macrophages was done in this study the data indicate that the differential chemokine production is due to different types of macrophages.

These data show that the classical and the non-classical monocytes in man may be committed to become unique types of macrophages. As to whether this concept also hold true for intermediate monocytes has not been tested as yet.

Using GM-CSF and IL-4 treatment monocytes can also be differentiated in vitro into cells with features of dendritic cells (Sallusto and Lanzavecchia 1994) and this strategy has been used widely to generate antigen-presenting cells for experimental vaccination against cancer. A comparative study on monocyte subsets has shown lower levels of CD1a and lower production of IL-12 in the DC derived from CD16-positive monocytes (Sanchez-Torres et al. 2001). Using the same approach Balboa et al. reported that DCs derived from CD16-positive monocytes are DC-SIGN negative when treated with GM-CSF and IL-4 while classical monocytes will give rise to DC-SIGN-positive DCs (Balboa et al. 2013). This difference was attributed to p38 MAP-kinase since levels of phosphorylated p38 were shown to be higher in the CD16-positive monocytes and blockade with a specific inhibitor led to development of DC-SIGN-positive DC from CD16-positive monocytes (Balboa et al. 2013). Taken together it appears that the non-classical monocytes in man are committed to become DCs which are less potent in antigen presentation.

In the mouse model it was shown that adoptive transfer of classical monocytes can give rise to DCs in the intestine and in the lung (Varol et al. 2007). Looking at lung DCs in more detail it was shown that classical monocytes give rise to CD103-positive DCs while non-classical monocytes can become CD103-negative DCs with a unique chemokine expression pattern (Jakubzick et al. 2008). Hence also in the mouse the monocyte subsets can differentiate into different monocyte-derived DCs.

1.5 Disease

1.5.1 General Remarks

Increased and decreased numbers of monocytes in blood during disease is mentioned in medical text books in various conditions but this was never central to diagnosis and management of patients. This is now about to change since with the

delineation of monocyte subsets informative difference can be detected in various clinical settings as given below. Most prominent here are the changes in the CD16-positive monocyte subset and their correlation with inflammatory markers.

Also, after the existence of the intermediate monocytes had been highlighted in the 2010 nomenclature proposal a wave of reports on increases of these cells has been published. Furthermore, the enumeration of DR-positive monocytes has been studied extensively for its prognostic values in various severe inflammatory diseases.

What has to be considered when evaluating monocytes in blood is the impact of age and gender. Here lower absolute numbers for total monocytes and for the CD16-positive monocytes have been reported for females (Heimbeck et al. 2010; Hearps et al. 2012a).

Also, CD16-positive monocytes increase with age (Seidler et al. 2010; Nyugen et al. 2010). The age-associated increase of both intermediate and non-classical monocytes was shown to go along with increased levels of TNF production (Hearps et al. 2012a).

When in different clinical settings the number of monocytes and their subsets show for instance an increase then this can indicate (a) that the total pool of these cells in the body expands and this is what we most frequently assume. However, the increase can also be due to (b) an increased influx from compartments like bone marrow, spleen or the marginal pool or (c) a reduced efflux (reduced emigration, reduced apoptosis). The same deliberations apply to decreases of monocytes. Therefore the increases and decreases always have to be interpreted with caution within the pathophysiological context.

1.5.2 Cancer

For cancer an early report by Saleh et al. noted a fourfold increase CD16-positive monocytes in gastric and colorectal malignancy (Saleh et al. 1995). In cholangiocarcinoma a threefold increase was reported and increased CD16-positive monocytes went along with higher density of tumor-associated macrophages (Subimerb et al. 2010). Also higher numbers of CD16-positive blood monocytes were associated with a worse prognosis. A twofold increase of CD16-positive monocytes was found in early stage breast cancer (Feng et al. 2011). Also in another study on colorectal cancer a twofold increase of CD16-positive intermediate monocytes was shown (Schauer et al. 2012). Macrophages in the past have been considered to have anti-tumor activity via direct cytotoxicity while more recently pro-tumor activity via provision of angiogenic factors has been favored. Whether the increased CD16-positive monocytes in cancer have a role in either of these processes is unclear. One attractive possibility for their expansion, however, is that their induction is secondary to cancer and due to tumor-derived factors, a concept supported by the finding that these cells decrease with tumor resection (Subimerb et al. 2010).

1.5.3 Infection and Inflammation

Sepsis is the most dramatic form of infection and here monocytes and macrophages and the cytokines they produce are considered important players. A strong increase of the CD16-positive monocytes has been demonstrated in such patients (Fingerle et al. 1993; Blumenstein et al. 1997; Shalova et al. 2012) and these expanded cells were shown to produce high levels of cytokines (Shalova et al. 2012). This indicates that the non-classical monocytes may contribute to the cytokine storm seen in the early phase of sepsis.

An increase of CD16-positive monocytes was also seen in severe bacterial infection in hemodialysis patients (Nockher and Scherberich 1998) and in patients with erysipelas, a skin infection with beta-hemolytic streptococci group A bacteria (Horelt et al. 2002). In the latter study lower levels of TNF were produced by the non-classical monocytes of patients as compared to controls. This may represent a state of unresponsiveness or tolerance, which is also seen in later stages of severe infections like sepsis. Such a tolerance in response to bacterial infection has been called compensatory anti-inflammatory response syndrome (CARS) and has been associated with a high mortality of patients (Bone et al. 1997). Severe sepsis can go along with a pronounced down-regulation of HLA-DR cell surface expression on blood monocytes (Volk et al. 1996). The effect is most pronounced for the classical monocytes, which show a fivefold reduction of HLA-DR expression compared to a twofold reduction for the non-classical monocytes in a group of abdominal aortic surgery patients (Kim et al. 2010b). The down-regulation of HLA-DR on monocytes can be detected in many other conditions like severe burn (Venet et al. 2007). Several studies have shown that low HLA-DR on blood monocytes correlates with higher mortality (Wu et al. 2011) while others failed to find such a correlation (Perry et al. 2003). Still with standardization (Docke et al. 2005) this parameter has potential as a predictor of clinical outcome in patients with sepsis, trauma, and immunosuppressive therapy.

In hemolytic uremic syndrome total monocytes can show a pronounced increase and here the CD16-monocytes expand (Fernandez et al. 2005) and this goes along with a decrease of HLA-DR on classical monocyte in the acute phase.

High levels of CD16-positive monocytes were also seen post cardiac surgery (Fingerle-Rowson et al. 1998a). Also, accidental blunt trauma was shown to lead to an expansion of intermediate monocytes and a correlation with inflammatory markers was noted (West et al. 2012). Here trauma-induced translocation of bacteria and/or LPS from the gut is a likely mechanism involved in the alterations of blood monocytes.

In addition, changes in monocyte composition have been noted for a whole spectrum of infectious diseases and the increases of CD16-positive monocytes in these conditions appear to be a recurrent pattern (Ziegler-Heitbrock 2007).

1.5.4 Infection of Monocytes by Viruses

Monocytes can respond to infection but they also can be targeted by microbes, especially by viruses. Merkel cell polyomavirus has been implicated in Merkel cell carcinoma, a neuroendocrine skin carcinoma. The virus was detected in blood leukocytes and here exclusively in the classical and not in the non-classical monocytes (Mertz et al. 2010).

With respect to Dengue virus infected patients show viral antigen in CD14-positive and CD16-positive cells in blood (Durbin et al. 2008). In vitro both classical and non-classical monocytes can be readily infected (Azeredo et al. 2010), however, only the CD16-positive monocytes responded with production of the pro-inflammatory cytokines IL-1 and TNF (Wong et al. 2012).

For Hepatitis C viral RNA was detected in CD16-positive but not in CD16-negative monocytes (Coquillard and Patterson 2009). This selective tropism was attributed to the higher expression of the CD81-HCV receptor on CD16-positive monocytes.

HIV infects T helper cells but also monocytes macrophages by using CD4 as receptor and either CXCR4 or CCR5 as co-receptors. Looking at blood monocytes HIV DNA can be detected in both classical and CD16 positive monocytes but the majority of patients shows an exclusive infection of the CD16-positive monocytes (Jaworowski et al. 2007). Furthermore, a higher viral load was noted for the CD16-positive monocytes in acute HIV infection (Centlivre et al. 2011).

The infection of monocytes by these viruses can result in further spreading in the body once the monocytes migrate into the different tissues.

1.5.5 Autoimmune Disease

Autoimmune disease, including rheumatoid arthritis and immune thrombocytopenia, is thought to be driven by specific responses of T and B cells to auto-antigens. Still auto-reactive lymphocytes can exist in apparently healthy individuals indicating that additional elements are required for disease to develop. Here an inflammatory milieu provided by monocytes–macrophages might be a critical element. For rheumatoid arthritis many alterations of these cells have been reported over the years (Kinne et al. 2000). For the blood monocytes increases of CD16-positive cells have been noted (Baeten et al. 2000; Cairns et al. 2002; Kawanaka et al. 2002). These findings may be compromised at times because patients are being treated with glucocorticoids, which are known to deplete the CD16-positive monocytes (Fingerle-Rowson et al. 1998b). More recently intermediate monocytes were shown to be expanded in RA and these cells were linked to increased development of Th17 cells in the disease (Rossol et al. 2012). These data suggest a new mechanism by which monocytes may impact on the inflammatory response in autoimmune disease. Also in the mouse model Lupus-prone animals show a strong increase of non-classical monocytes (Santiago-Raber et al. 2009).

In immune thrombocytopenia CD16-positive monocytes are also expanded and their number increase with the decrease of the platelet count (Zhong et al. 2012). Here high CD16-positive monocytes went along with high numbers of T helper 1 (TH1) cells and with low Treg and Th17 cells. The latter effect was shown to be due to IL-12 cytokine likely produced by the CD16-positive monocytes. These findings demonstrate yet another scenario, in which monocytes interact with T cells in order to promote autoimmunity.

The contribution of monocytes to autoimmunity may also be more direct in that the monocyte Fc-receptor binds the autoantibody and thereby can target and destroy self, like for instance autologeous platelets. In a mouse model for ITP using a monoclonal anti-platelet antibody Biburger et al. could show that the non-classical monocytes are crucial to platelet depletion (Biburger et al. 2011). These data demonstrate a direct role for monocytes in autoimmune disease for this model system.

1.5.6 Liver Disease

In acute liver failure, characterized by coagulopathy and encephalopathy, blood monocytes show a shift within the CD16-positive monocytes in that the intermediate monocytes increase and the non-classical monocytes strongly decrease (Abeles et al. 2012). One explanation for the decrease of non-classical monocytes in blood is that these cells may migrate into the severely damaged liver tissue. Also in acute liver failure a pronounced down-regulation of HLA-DR expression on CD14-positive blood monocytes has been noted and this was associated with poor prognosis (Antoniades et al. 2006).

In fibrosis (cirrhosis) of the liver caused by chronic inflammation the CD16-positive monocytes are increased in blood (Zimmermann et al. 2010) and there also is a strong increase of CD16-positive macrophages in liver tissue as shown by immunohistology. This accumulation may be due in part to increased transmigration of the CD16-positive monocytes across hepatic sinusoidal endothelial cells (Aspinall et al. 2010).

When isolating human leukocytes from liver by mechanical disruption, then monocytes can be isolated, which apparently reside in the hepatic sinusoids and here the intermediate monocytes comprise 1/3 of all monocytes in the normal and diseased liver (Liaskou et al. 2013). In addition to the increase of CD16-positive blood monocytes in cirrhosis a pronounced rise in the soluble IL-2 receptor (sCD25) can be observed (Seidler et al. 2012). Since the CD16-positive monocytes show a higher expression level for this receptor they are a likely source, but tissue macrophages can also be CD25 positive and may release this receptor as has been shown for inflammatory lung disease (Pforte et al. 1993).

While in man the CD16-positive monocytes predominate in liver fibrosis (Zimmermann et al. 2010), the mouse model of CCL4 induced liver fibrosis is dominated by the classical monocytes (Karlmark et al. 2009). The CCL4 induced

fibrosis in the mouse is, however, a short term model, which may not adequately reflect the processes occurring over many years in human liver cirrhosis (Tacke 2012).

In any event it has become clear over the last couple years that monocytes and their macrophage progeny are central to inflammation and fibrosis of the liver.

1.5.7 Cardiovascular Disease

1.5.7.1 Atherosclerosis

In atherosclerosis for many decades lipids and lipoproteins have been considered the major culprits in the pathophysiology of the disease (Glass and Witztum 2001). The leukocyte infiltration in the vascular lesions, however, demonstrated that atherosclerosis also is an inflammatory process (Ross 1999). Since macrophages form a major part of the inflammatory infiltrate the role of these cells and of the blood monocytes has taken center stage. The importance of these cells is demonstrated by the reduced pathology in atherosclerosis-susceptible mice when they have at the same time a M-CSF defect with strongly reduced monocyte/macrophage numbers (Smith et al. 1995). Further support comes from the finding that clodronate-mediated depletion of monocytes in rabbits fed a high cholesterol diet will decrease the number of atherosclerotic lesions (Ylitalo et al. 1994). Oxidated LDL may be crucial for atherosclerosis by attracting monocytes into arterial vessel wall in a process involving up-regulation of endothelial adhesion molecules and chemokines. The essential role of adhesion molecules has been demonstrated by the reduced atherosclerosis in mice with defects of ICAM1 and P-selectin (Nageh et al. 1997). Also, multiple chemokines appear to be involved in this process since knock-out of the CCR2, CCR5, and CX3CR1 chemokine receptors have additive effects in blocking atherosclerosis in the mouse model (Combadiere et al. 2008). Once arrived in the lesion monocytes can develop into macrophages and can become lipid laden foam cells. The local macrophages can produce cytokines and chemokines, which orchestrate the local inflammatory process. The plaque is a dynamic structure and involves egress and continuous influx of monocytes, such that blockade of influx will lead to resolution of the lesion (Potteaux et al. 2011).

While it is clear that monocytes are crucial to atherosclerosis the number of total monocytes has no predictive value for cardiovascular events in atherosclerosis patients (Wheeler et al. 2004). However, an analysis of monocytes subsets may reveal a different picture.

When looking at monocyte subsets in ApoE$^{-/-}$ mice on a high fat diet, then these atherosclerosis-prone animals show a pronounced increase of monocytes with the strongest effect on the classical monocytes (Tacke et al. 2007; Swirski et al. 2007). However, tracking studies revealed that both classical and non-classical subsets enter the atherosclerotic plaque (Tacke et al. 2007). Intravital multiphoton microscopy can demonstrate a substantial accumulation of non-classical mouse monocytes

in the lesions and these can be reduced by statin treatment (Haka et al. 2012). Studies on monocyte subsets in atherosclerosis in man looked at patients with familial hypercholesteremia, who have a defect of the LDL receptor and are at high risk of developing atherosclerosis. In these patients the CD16-positive monocytes were shown to preferentially take up oxLDL (Mosig et al. 2009). At the same time the CD16-positive monocytes are strongly reduced in FH and it was speculated that this is due to increased adherence to vascular endothelium followed by migration into the blood vessel wall. In patients with atherosclerosis manifesting as coronary heart disease an increased number of CD16-positive cells was reported and found to be associated with increased CRP (Huang et al. 2012). Hence this subset is associated with a main risk factor for cardiovascular events in atherosclerosis (Ridker 2007). When taking a closer look at the CD16-positive monocytes then the intermediate cells are predictors of cardiovascular events in at risk dialysis patients and this is independent of CRP (Heine et al. 2008).

CD16-positive monocytes were shown to positively correlate with vulnerable plaques in coronary heart disease patients (Kashiwagi et al. 2010). Also these cells were shown to be linked to intima media thickness and to obesity (Poitou et al. 2011). Rogacev et al. demonstrated that it is the intermediate $CD14^{++}CD16^{+}$ monocytes, which are predictive for cardiovascular events in such patients (Rogacev et al. 2012).

1.5.7.2 Post-cardiovascular Events

In a mouse model using cryoinjury of the heart muscle the depletion of monocytes led to higher mortality and reduced ventricular function (van Amerongen et al. 2007) indicating that these cells are necessary for proper wound healing and reconstitution of heart function. On the other hand in myocard infarct (MI) patients a high monocyte count in the days after the event correlated with subsequent heart failure and ventricular aneurysm, i.e., high numbers are detrimental (Maekawa et al. 2002).

When looking at monocyte subsets in the mouse in the first days post MI the classical monocytes were shown to migrate into the infarct while non-classical monocytes infiltrated the tissue beginning on day 5 post MI (Nahrendorf et al. 2007). In this model the classical and non-classical subsets were shown to go along with inflammatory and reparative functions, respectively.

In man the patterns of monocyte subpopulations post MI are consistent with these mouse data. In human blood the classical monocytes peaked on day 3 and decreased thereafter while non-classical monocytes peaked on day 5 and remained high (Tsujioka et al. 2009). The same study demonstrated that high numbers of classical monocyte were associated higher damage as reflected in reduced salvage and reduced left ventricular ejection. Also, high numbers of classical monocytes in blood correlated with higher degrees of vascular occlusion in patients post MI (Tsujioka et al. 2010).

Conversely, in MI patients those without ventricular thrombus formation had higher non-classical monocytes in blood suggesting a protective effect (Frantz et al. 2013).

Taken together while both monocyte subsets are required for the post MI repair process, it appears that classical monocytes may be detrimental and non-classical may be beneficial when it comes to repair and long term organ function. The role of intermediate monocytes in this process remains to be determined.

1.5.7.3 Development of Collateral Blood Vessels

After occlusion of an artery blood supply to the affected tissue can be reconstituted by the growth of new blood vessels, which are called collaterals. Macrophages are seen in areas of collateral growth and monocyte/macrophage deficient op/op mice show a reduced development of collaterals after femoral occlusion in the mouse model (Bergmann et al. 2006). Adoptive transfer experiments demonstrated an increased angiographic score for both classical and non-classical monocytes but only the classical monocytes improved capillary density (Cochain et al. 2010). It is conceivable that angiogenic factors generated by monocytes contribute to collateral growth after arterial occlusion and here TIE2-positive intermediate monocytes (Zawada et al. 2011; De Palma et al. 2005) may be important players.

1.5.8 Kidney Disease

Patients with chronic renal failure on hemodialysis show increased numbers of CD16-positive monocytes (Nockher and Scherberich 1998; Heine et al. 2012) and this is also true for patients on peritoneal dialysis (Saionji and Ohsaka 2001). This increase may reflect a state of chronic microinflammation in these patients.

During hemodialysis a decrease in neutrophils has been reported already in the 1960s (Kaplow and Goffinet 1968). A decrease in the monocyte count was seen for cuprophane dialysers with less of an effect for biocompatible polyacrylonitrile membranes (Stuard et al. 1995). Even biocompatible membranes show an effect on monocytes in that there is a preferential decrease of the CD16-positive cells (Nockher et al. 2001; Sester et al. 2001). The decrease is not seen when the blood recirculates in the dialyzer only, but it requires the blood to reenter the circulation (Sester et al. 2001). Therefore, the decrease in leukocytes is thought to be triggered by the interaction with the dialyzer membranes followed by adhesion of the cells to vascular endothelium once the blood is re-infused. The data suggest that the decrease of CD16-positive monocytes may serve as a sensitive indicator of biocompatibility.

1.5.9 Immunodeficiency

There are two aspects of monocytes in immunodeficiency (a) alterations of monocytes in defects of other parts of the immune system (notably T and B cells) and (b) defects of the monocytes themselves.

(a) Alterations of Monocytes in T and B Cell Defects

Here higher numbers of CD16-positive monocytes were noted in B-cell deficient patients with a mutation of Bruton's tyrosine kinase (Btk) (Amoras et al. 2007). In patients with common variable immunodeficiency (CVID) a slight increase was noted for the intermediate monocytes, while in the same study an increase of the non-classical monocytes was confirmed for patients with Btk defects (Barbosa et al. 2012). The expansion of these cells may reflect an inflammatory reaction in patients, who with their antibody defect may have smouldering bacterial infection. Here the moderate antibody defect in CVID may lead to a weaker signal such that only intermediate monocytes increase. By contrast, the almost complete antibody defect in Btk-defective patients may lead to more pronounced bacterial infections and thereby can provide a stronger signal leading to further differentiation and increase of non-classical monocytes.

A prominent defect of CD4 helper T cells is seen in HIV infection. An increase of CD16-positive monocytes in these patients has been noted in many studies (Allen et al. 1991; Locher et al. 1994; Ancuta et al. 2008; Hearps et al. 2012b; Han et al. 2009; Kamat et al. 2012). A similar scenario as discussed above for antibody deficiency may apply to patients with HIV infection in that smouldering opportunistic infection may trigger the expansion of CD16-positive monocytes. Also, a role for bacterial translocation from the gut leading to detectable LPS levels in blood has been discussed to impact on monocyte subsets in AIDS patients (Ancuta et al. 2008; Funderburg et al. 2012). Patients with a high viral load showed the strongest increase of non-classical monocytes (Funderburg et al. 2012). Of note, these changes may also be influenced by the infection of the CD16-positive monocytes by the HI virus as discussed above (Jaworowski et al. 2007; Centlivre et al. 2011).

(b) Monocytes Defects

In the mouse model a decrease in blood monocytes was noted in the op/op mouse, which is characterized by a defect of the M-CSF gene and this has multiple effects on the monocyte–macrophage system (Wiktor-Jedrzejczak et al. 1982; Yoshida et al. 1990). Most notable is the reduction in osteoclast number and activity leading to osteopetrosis but monocyte numbers are also severely reduced in these animals.

A threefold reduction of classical monocytes is seen in the CCR2 knock-out animals in which these monocytes are retained in the bone marrow (Serbina and Pamer 2006). Such animals breed normally but upon intravenous injection of Listeria monocytogenes they are unable to clear infection, apparently because the recruitment of monocytes to the tissue is impaired (Kurihara et al. 1997). Reduced monocyte numbers have also been noted in several additional knock-out models. Kruppel-Like Factor $4^{-/-}$ mice show lower blood monocytes and here the classical monocytes are reduced tenfold while the non-classical are reduced twofold (Alder et al. 2008). Also, $p21CIP1^{-/-}$ mice show a mild reduction on blood monocytes mainly due to the decrease of classical monocytes (Scatizzi et al. 2006). Finally, $Nur77^{-/-}$ mice show a moderate decrease of blood monocytes and this is due to a selective decrease of the non-classical monocytes (Hanna et al. 2011; Chao et al. 2013).

Among these models the strongest reduction of blood monocytes is seen in the op/op mice. Still even these animals do not have a strong immunodeficiency phenotype; while half of the animals die during weaning and while the remainder dies after about 6 months; there is no need to keep animals under sterile conditions as is the case for T cell deficient mice. This indicates that monocytes/macrophages are not crucial to immune defense under standard housing conditions or alternatively that a few remaining monocytes/macrophages are sufficient to control natural microbial infection.

In man only a few reports on clinical cases with deficient monocyte numbers have been reported recently. Mutations of the GATA2 transcription factor were shown to lead to a defect of NK-cells, B cells, and monocytes (Vinh et al. 2010; Bigley et al. 2011; Hsu et al. 2011). Furthermore, mutations of the transcription factor IRF8 lead to a defect in circulating DCs and monocytes (Hambleton et al. 2011). These two types of mutations lead to a defect in both classical and non-classical monocytes. A selective defect of the CD16-positive monocytes (i.e., non-classical and intermediates) was reported for a family with three affected siblings (Frankenberger et al. 2013). One sibling suffered from an undefined auto-inflammatory disease, while the other two were apparently healthy. This indicates that the function of the CD16-positive blood monocytes can be compensated by other parts of the immune system. The genetics of this defect remain to be elucidated and will depend on additional informative families.

An overview of the clinical conditions associated with increased or decreased numbers of CD16-positive monocytes is given in Table 1.1.

Table 1.1 Conditions showing increases and decreases of CD16-positive monocytes

Increased	Subset[a]	Species	Reference
Male	CD16-positive	Man	Heimbeck et al. (2010), Hearps et al. (2012a)
Age	Intermediate/ non-classical	Man	Seidler et al. (2010), Fehlings and Nguyen (2010), Hearps et al. (2012a)
Exercise	CD16-positive	Man	Steppich et al. (2000)
Colorectal cancer	CD16-positive	Man	Saleh et al. (1995)
	Intermediate	Man	Schauer et al. (2012)
Cholangiocarcinoma	CD16-positive	Man	Subimerb et al. (2010)
Breast cancer	CD16-positive	Man	Feng et al. (2011)
Infection (sepsis)	CD16-positive	Man	Fingerle et al. (1993), Blumenstein et al. (1997), Pachot et al. (2008), Shalova et al. (2012)
Infection (bacterial)	CD16-positive	Man	Nockher and Scherberich (1998), Horelt et al. (2002)
Trauma	CD16-positive	Man	Fingerle-Rowson et al. (1998a)
	Intermediate	Man	West et al. (2012)

(continued)

Table 1.1 (continued)

Increased	Subset[a]	Species	Reference
Rheumatoid arthritis	CD16-positive	Man	Baeten et al. (2000), Cairns et al. (2002), Kawanaka et al. (2002)
	Intermediate	Man	Rossol et al. (2012)
Lupus erythematodes	Non-classical	Mouse	Santiago-Raber et al. (2009)
Immune thrombocytopenia	CD16-positive	Man	Zhong et al. (2012)
Liver failure	Intermediate	Man	Abeles et al. (2012)
Liver fibrosis	CD16-positive	Man	Zimmermann et al. (2010)
Atherosclerosis	CD16-positive	Man	Huang et al. (2012), Kashiwagi et al. (2010), Rogacev et al. (2010)
Obesity	Intermediate/ non-classical	Man	Rogacev et al. (2010) Poitou et al. (2011)
On hemodialysis	CD16-positive	Man	Nockher and Scherberich (1998), Saionji and Ohsaka (2001)
HIV infection	CD16-positive	Man	Allen et al. (1991), Locher et al. (1994), Ancuta et al. (2008), Hearps et al. (2012b), Han et al. (2009), Kamat et al. (2012)
CVID	Intermediate	Man	Barbosa et al. (2012)
BTK-defect	Non-classical	Man	Barbosa et al. (2012), Amoras et al. (2007)
M-CSF	Intermediate/ non-classical	Man	Munn et al. (1990), Weiner et al. (1994)
TLR9L	Intermediate/ non-classical	Mouse	Santiago-Raber et al. (2011)
TLR7/8/9 L	Intermediate/ non-classical	Primates	Kwissa et al. (2012)

Decreased	Subset[a]	Species	Reference
Familial hypercholesterolemia	CD16-positive	Man	Mosig et al. (2009)
During hemodialysis	CD16-positive	Man	Nockher et al. (2001), Sester et al. (2001)
GATA2, IRF8 defect	All monocytes	Man	Vinh et al. (2010), Hambleton et al. (2011)
Genetic defect	Intermediate/ non-classical	Man	Frankenberger et al. (2013)
M-CSF defect	All monocytes	Mouse	Wiktor-Jedrzejczak et al. (1982), Yoshida et al. (1990)
NUR 77 defect	Non-classical	Mouse	Hanna et al. (2011), Chao et al. (2013)
KFL-4 defect	Non-classical	Mouse	Alder et al. (2008)
Glucocorticoids	CD16-positive	Man	Fingerle-Rowson et al. (1998b), Dayyani et al. (2003), Fertl et al. (2008)
M-CSF blockade	Non-classical	Mouse	MacDonald et al. (2010), Lenzo et al. (2012)
	CD16-positive	Man	Korkosz et al. (2012)
IgG infusion	CD16-positive	Man	Katayama et al. (2000), Siedlar et al. (2011)
Apheresis	CD16-positive	Man	Hanai et al. (2008), Kanai et al. (2007)

[a]The term "CD16-positive" denotes that the intermediate and non-classical subsets have not been looked at separately in the respective study

1.6 Therapy

Depletion of blood monocytes and their subsets has been observed with several therapeutic approaches and this includes glucocorticoids, high dose chemotherapy, intravenous IgG infusion, clodronate liposomes, blockade of the CCL2 pathway and of the M-CSF pathway.

A depletion of blood monocytes cannot be expected to have a strong effect of its own given the limited role of these cells in immune defense within the blood compartment. However, their depletion may result in the blockade of replenishment of tissue macrophages from immigrating monocytes under homeostatic and inflammatory conditions. Also, a depletion of blood monocytes may go along with a similar depletion of tissue macrophages via a direct effect of the treatment on these cells. In any event, the blood monocytes can serve as a useful indicator of the systemic effects of a given compound.

Glucocorticoids (GCs) have many anti-inflammatory modes of action and their depleting activity regarding monocytes became only apparent after the discovery of the CD16-positive monocytes. Intravenous GCs but also oral GCs can deplete the non-classical monocytes within a few days while the classical monocytes increase at the same time (see Fig. 1.4) (Fingerle-Rowson et al. 1998b; Dayyani et al. 2003; Fertl et al. 2008; Heimbeck et al. 2010).

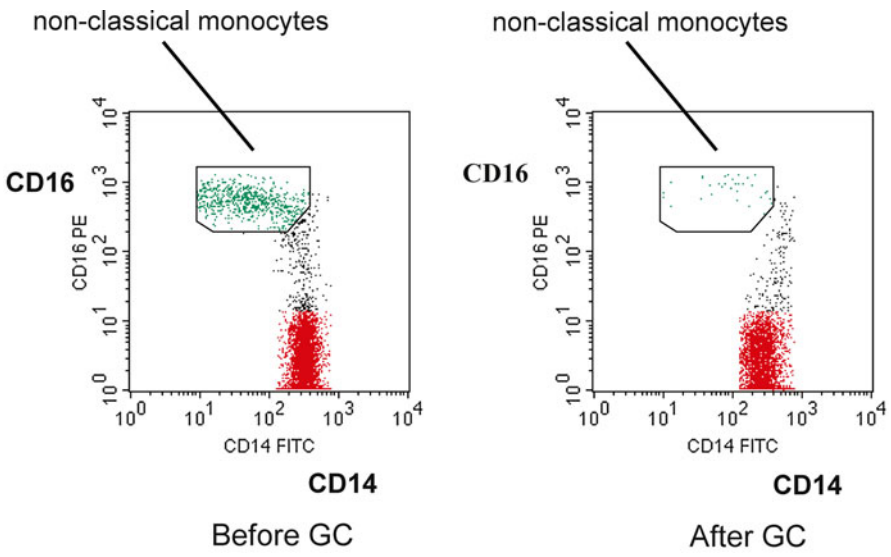

Fig. 1.4 Decrease of non-classical monocytes after high dose glucocorticoid therapy. Non-classical monocytes were reduced from 27 to 2 cells/μL 3 days after a daily dose of methylprednisolone at 500 mg. (Taken from (Dayyani et al. 2003) with permission of the publisher)

Also, high dose chemotherapy will deplete all monocytes and the reconstitution starts with the classical monocytes followed by the non-classical monocytes (Dayyani et al. 2004).

High dose intravenous IgG (IVIG) is used as therapy for Kawasaki disease, a pediatric inflammatory disease of unknown etiology. Infusion of 400 mg/kg per day for 5 days was shown to go along with a substantial fivefold decrease of CD16-positive monocytes (Katayama et al. 2000). Also a single dose of 400 mg of IgG/kg used as substitution for adult patients with common variable immunodeficiency will reduce non-classical monocytes 2.5-fold (Siedlar et al. 2011). In addition this treatment leads to a reduction in TNF production by the monocytes. The data suggest that the anti-inflammatory action of IVIG-therapy may operate via its effects on blood monocytes.

Clodronate encapsulated in liposomes has been used widely in experimental animals to deplete blood monocytes and macrophages by inducing apoptosis (van Rooijen and Hendrikx 2010). This has become a valuable tool for the study of different types of macrophages dependent on the route of application. Intravenous injection will deplete blood monocytes within 24 h (Sunderkotter et al. 2004) and it will also target macrophages in the liver and in the spleen. This strategy has, however, not been developed further for application in man.

Apheresis of leukocytes has been used as a therapy for otherwise refractory diseases like ulcerative colitis and psoriasis pustulosa. Passage of whole blood through apheresis columns will deplete neutrophils and at the same time monocytes are reduced. Among the monocytes there is a preferential depletion of the CD16-positive as compared to classical monocytes (Hanai et al. 2008; Kanai et al. 2007). Similar findings have been reported for psoriasis (Fujisawa et al. 2012). Patients on glucocorticoids show lower levels of CD16-positive monocytes and apheresis will lead to a further decrease (Hanai et al. 2008). Patients responding clinically to apheresis show a stronger decrease of the CD16-positive monocytes (Iwakami et al. 2009). The preferential decrease of CD16-positive monocytes during apheresis is reminiscent of what is seen upon hemodialysis, where a selective decrease of these monocytes also has been observed (Nockher et al. 2001; Sester et al. 2001). The similar behavior of the CD16-positive monocytes in these two settings may be due to a higher adhesion propensity of these cells brought about by higher levels of cell surface adhesion molecules.

CCR2 is the chemokine receptor for CCL2 (MCP1) and this receptor is expressed by classical but not by non-classical monocytes (Weber et al. 2000). The classical CCR2-positive monocytes are central to models of autoimmune disease like, for instance, experimental encephalitis (Fife et al. 2000; Izikson et al. 2000). Treatment with anti-CCR2 can deplete the classical monocytes and show beneficial effects at low doses of the antibody but at high doses additional effects on other cells like mast cells can counteract the beneficial effect of this monocyte depletion in arthritis models (Bruhl et al. 2007). Clinical trials with anti-CCR2 in man failed to show a therapeutic benefit in RA patients (Vergunst et al. 2008). Actions on CCR2-positive cells other than monocytes may explain this failure in part.

Studies using an anti-M-CSF-receptor antibody in the mouse model have shown depletion of the non-classical monocytes and tissue macrophages from many organs (MacDonald et al. 2010) but in inflammatory conditions no effect on peritoneal macrophage numbers was seen. Another M-CSF-R antibody also resulted in the depletion of non-classical Ly6C^{low} mouse monocytes and of macrophages in the lung and in the peritoneum and this was observed under homeostatic and inflammatory conditions (Lenzo et al. 2012). The question is whether these antibodies deplete tissue macrophages directly or whether these cells are depleted because their replenishment from the non-classical blood monocytes is blocked. In non-human primates an anti-M-CSF-antibody was shown to strongly reduce non-classical monocytes (US patent 7728113). Also in a pilot study it was demonstrated that treatment of rheumatoid patients with anti-M-CSF can deplete both non-classical and intermediate monocytes (Korkosz et al. 2012). These data are in line with the converse finding of an expansion of the intermediate and non-classical monocytes in patients treated with M-CSF (Munn et al. 1990; Weiner et al. 1994). An alternative approach to target monocytes via the M-CSF pathway involves the use of receptor-tyrosine-kinase inhibitors, which can block signaling via this pathway. Some of these inhibitors are already available for treatment of malignancies (Faivre et al. 2007). Their potential in the context of monocyte-directed intervention needs still to be explored.

Taken together there are several novel monocyte-directed therapies and these have potential for a wide spectrum of inflammatory diseases.

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Chapter 2
Polarized Activation of Macrophages

Maria Rosaria Galdiero, Subhra K. Biswas, and Alberto Mantovani

2.1 Introduction

The first line of defense against pathogens is constituted by the innate immune system. Several recent evidences also indicate innate immunity receptors as primary sensors of tissue damage and metabolic disorders (Medzhitov 2008; Verbist et al. 2013). The innate immune system comprises a cellular and a humoral arm. Mononuclear phagocytes such as macrophages belong to the cellular arm of innate immunity. These cells along with the dendritic cells play a pivotal role in initiating, orientating, and modulating many aspects of the adaptive response.

In addition to their role as innate effector cells, macrophages and neutrophils also represent a major source of humoral, fluid phase pattern recognition molecules (Bottazzi et al. 2010; Deban et al. 2010). Among them are the long pentraxin PTX3, members of the ficolin and collectin family, and serum amyloid A. Since they can be considered as functional ancestors of antibodies, the production of these soluble mediators by phagocytes bridges the gap between the cellular and the humoral arm of innate immunity (Bottazzi et al. 2010).

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Macrophages are found in almost all tissues and have important roles in immunity, tissue repair, metabolic homeostasis, and development (Biswas and Mantovani 2010, 2012; Gordon and Martinez 2010; Pollard 2009; Wynn et al. 2013). Phenotypic and functional plasticity is a key feature of macrophages (Mantovani et al. 2002, 2004; Martinez et al. 2009; Sica and Mantovani 2012; Mosser and Edwards 2008; Gordon and Taylor 2005; Biswas and Mantovani 2010). Under physiological conditions, macrophages modulate their morphological and functional aspects in response to their tissue microenvironment and give rise to distinct resident populations, such as Kupffer cells in the liver, alveolar macrophages in lungs, lamina propria macrophages in the gut, and microglia in the brain. Under pathological stimuli, such as microbes or tissue damage, macrophages activate their effector functions, namely antimicrobial and antitumoral activities. However, far from being only a transient increase in their effector functions, it is now clear that macrophage "activation" is characterized by several distinct aspects which give rise to macrophage phenotypes with distinct and specific roles (Mackaness 1969; Adams and Hamilton 1984; Evans and Alexander 1972). In this regard, the discovery of an IL-4 mediated "alternative" form of macrophage activation (Stein et al. 1992) has shed new light on the complexity of macrophage polarization. It is now widely accepted that, mirroring the Th1–Th2 paradigm and under the crucial influence of soluble mediators, namely IFN-γ or IL-4/IL-13, macrophages can be polarized toward a "classical" M1 or "alternative" M2 phenotype, respectively. However, M1 and M2 phenotypes are two extremes of a spectrum of functional states which make up the complexity of macrophage plasticity (Biswas and Mantovani 2010; Mantovani et al. 2002; Mosser and Edwards 2008; Sica and Bronte 2007; Sica and Mantovani 2012).

Here we will review the salient features of macrophage polarization as well as the underlying genetic and epigenetic mechanisms, following an overview of the new findings on macrophage origin and development.

2.2 Origin and Diversity of Macrophages

Tissue macrophages were classically viewed as terminally differentiated cells derived from circulating monocytes that originate in the bone marrow. This point of view has recently been challenged by increasing evidence that suggest tissue macrophages to originate prior to birth and maintained in the adulthood, independent of the contribution from monocytes (Wynn et al. 2013; Yona et al. 2013). In the mouse at least three distinct lineages of mononuclear phagocytes have been described: the first one derives from the yolk sac (YS) and gives rise to the tissue-resident populations of macrophages (defined as $F4/80^{high}$) in skin, spleen, pancreas, liver brain and lung; the second population originates from fetal liver and gives rise to Langerhans cells, with a minor contribution from YS; and the third one originates from the bone marrow and gives rise to circulating monocytes that differentiate into the $F4/80^{+}$ macrophage population within the tissues, e.g., lamina propria macrophages

(Schulz et al. 2012; Wynn et al. 2013; Ginhoux et al. 2010; Hoeffel et al. 2012). In some organs like kidney and lung, co-existence of macrophages derived from YS as well as circulating monocytes has been proposed. In the same vein, two distinct populations of Kupffer cells (KCs) have been identified: the first one, radiosensitive, bone marrow-derived, rapidly replaced from hematopoietic precursors upon reconstitution following irradiation reconstitution and a second one, radioresistant, termed "sessile" because of the absence of rapid turnover and the missing capacity for local recruitment. Only the first KC population was found to take part into inflammatory responses (Klein et al. 2007). Interestingly in the case of lung, a recent paper demonstrated fetal monocytes to give rise to alveolar macrophages (Guilliams et al. 2013). However, further studies will be required to clarify the relative contributions of YS and fetal liver in the origin of different tissue-resident macrophages.

Colony stimulating factor-1 (CSF-1) and its receptor (CSF-1R) is a major cytokine regulating the differentiation of macrophages (Chitu and Stanley 2006; Hamilton and Achuthan 2013). Accordingly, genetic ablation of CSF-1 or its receptor, CSF-1R results in the loss of macrophages in many tissues, such as skin, brain, bone, testis, and ovary. However compared to the CSF-1 deficiency, genetic ablation of CSF-1R resulted in a more severe phenotype, characterized by a complete lack of microglia, suggesting a role for another ligand of the CSF-1R (Erblich et al. 2011). This was identified as IL-34 (Wang et al. 2012; Lin et al. 2008; Wei et al. 2010). Indeed, genetic ablation of IL-34 had resulted in loss of microglia and Langerhans cells (Wang et al. 2012). A recent study on the transcriptional factors responsible for macrophage differentiation revealed an interesting dichotomy with YS-derived murine tissue-resident macrophages (such as microglia) to be dependent on CSF-1R and the transcription factor PU.1 but not Myb, while the bone marrow-derived macrophages being dependent Myb (Schulz et al. 2012). Other factors like GM-CSF has been shown to be critical to differentiation of alveolar macrophages (Guilliams et al. 2013) and the compensatory role of VEGF in osteoclast development (Niida et al. 1999).

In contrast to the steady-state differentiation of macrophages as described above, under pathological situation like inflammation, blood monocytes are a key source of inflammatory macrophages and inflammatory DCs. However, in murine models of Type 2 inflammation, macrophage accumulation was found to be maintained by local self-renewal, independently of replacement by circulating monocytes or other putative precursors (Jenkins et al. 2011; Liddiard et al. 2011; Davies et al. 2011). Interestingly, in the liver, KCs have also been suggested to be maintained by local proliferation, following hepatectomy (Widmann and Fahimi 1975). However, further studies are necessary to ascertain the relative role of monocyte-derived and local expansion of macrophages in other tissue compartments upon pathological settings. Lastly, considering the emerging differences between murine and human macrophages differ a lot from each other (Martinez et al. 2013), it will be interesting to explore the existence of multiple origins and the functional significance of distinct lineages of macrophages in human settings.

2.3 Macrophage Activation and Polarization

The concept of plasticity of mononuclear phagocytes became more complex when an alternative form of macrophage activation induced by IL-4 was found (Stein et al. 1992). Indeed, the Th1 cytokine, IFN-γ alone or together with microbial stimuli (e.g., LPS) or inflammatory cytokines (e.g., TNF and GM-CSF) was the first soluble mediator found to activate classical effector functions of macrophages (Fig. 2.1). IL-4 and IL-13 were subsequently found to be responsible for an "alternative" (M2) form of macrophage activation (Gordon 2003) (Fig. 2.1). The term M1 and M2 was initially proposed to describe macrophage populations that showed distinct nitrogen metabolism pathways (Nitric oxide versus arginine) upon LPS or IFNγ stimulation, depending on whether they were derived from Th1 mice strains (e.g., C57/BL6) or Th2 mice strains (e.g., Balb/c) (Mills et al. 2000). This concept was extended and further developed by Mantovani and colleagues to propose a general scheme for macrophage polarization, wherein M1 state represented the classically activated macrophages, whereas the M2 state included the alternatively activated macrophages (Mantovani et al. 2002). It is now known that other mediators besides IL-4 and IL-13 can also drive M2 polarization. For example, IL-33, a cytokine of the IL-1 family (Hazlett et al. 2010; Kurowska-Stolarska et al. 2009), amplifies IL-13-induced polarization of alveolar macrophages to an M2 phenotype which is responsible for lung eosinophilia and inflammation (Kurowska-Stolarska et al. 2009). Similarly, IL-21 is another Th2-associated cytokine that is shown to promote M2 activation of macrophages (Pesce et al. 2006). CSF-1 and IL-34 have also been suggested to polarize macrophages to a M2 phenotype (Foucher et al. 2013; Martinez et al. 2006). Indeed, a study of the LPS response of GM-CSF and CSF-1 derived bone marrow macrophages showed the former to induce more IL-12 and IL-23 resembling an M1 state while the latter induced more of IL-10 but no IL-12/23, suggesting a M2 polarization (Fleetwood et al. 2007). Activin A was found to be one of the molecules responsible for the M1 polarization of GM-CSF-derived macrophages (Sierra-Filardi et al. 2011).

In addition, among the various activation states that characterize the macrophage complexity is an "M2-like" state, which shares some but not all the functional aspects of M2 cells (Biswas and Mantovani 2010) (Fig. 2.1). Various stimuli, such as immunoglobulin complexes, glucocorticoids, transforming growth factor-β (TGF-β), and IL-10, give rise to M2-like functional phenotypes that exhibit properties similar to IL-4- or IL-13-activated macrophages (e.g., high expression of mannose receptor, IL-10, and angiogenic factors) (Mantovani et al. 2004). In addition, many in vivo conditions were found to be characterized by the appearance of M2-like macrophages, such as in the placenta and embryo, helminth or *Listeria* infection, obesity, and cancer (Auffray et al. 2007; Gustafsson et al. 2008; Odegaard et al. 2007; Rae et al. 2007; Raes et al. 2005).

From the functional point of view, M1 cells play a pivotal role in polarized Th1 responses and mediate resistance against intracellular parasites and tumors. In fact, these cells produce high levels of IL-12 and IL-23, as well as effector molecules

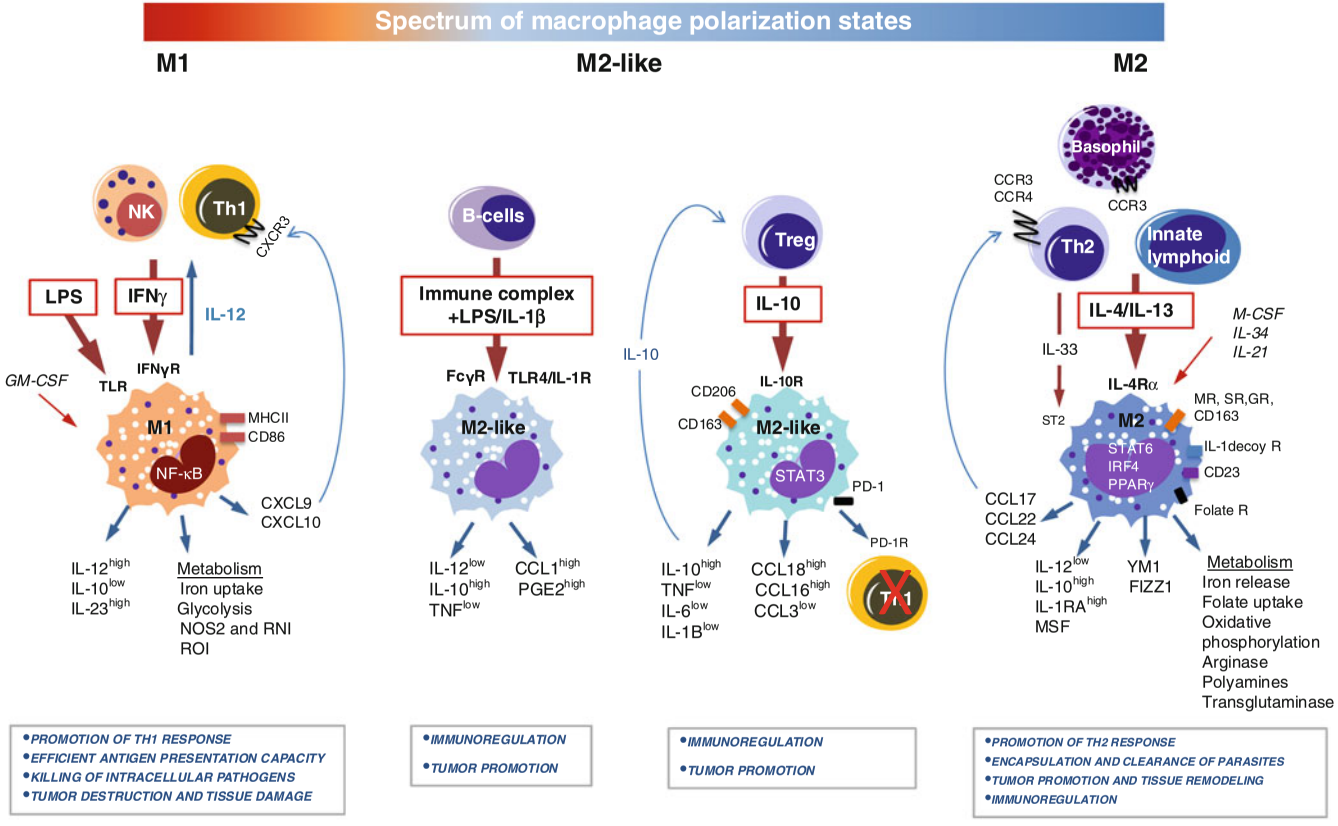

Fig. 2.1 Polarized activation of macrophages. Figures showed the salient features of the M1, M2, and M2-like polarized macrophages. For each polarization state, key genes, metabolic features, transcription factors, and functional properties associated with it are indicated. The principal stimuli responsible for each polarizing state are indicated by the *red box* (color figure online). Other polarizing stimuli are shown in *italics*. Crosstalk between polarized macrophage and lymphocyte subsets is also shown. Figure is adapted from Biswas and Mantovani (2010)

(e.g., reactive nitrogen and oxygen intermediates, RNI, ROI) and inflammatory cytokines (IL-1β, TNF, IL-6), but low levels of the immunoregulatory cytokine IL-10. On the contrary, the various forms of M2 macrophages generally express high levels of IL-10, low levels of IL-12 and IL-23, and display variable capacity to produce inflammatory cytokines. M2 cells are generally characterized by high expression of scavenger, mannose, and galactose-type receptors as well as Arginase-1, which is responsible for the production of ornithine and polyamines. M2 cells also express low levels of IL-1β and caspase I, high levels of IL-1ra and decoy type II receptors (Dinarello 2005). In terms of function, M2 cells are mainly involved in polarized Th2 responses, such as parasite clearance (Noel et al. 2004). In addition, these cells along with M2-like macrophages display immunoregulatory properties, promote tissue remodeling, angiogenesis, and tumor progression (Wynn 2004; Mantovani et al. 2013; Biswas and Mantovani 2010).

Chemokine production and chemokine receptor expression are also differentially represented by M1- and M2-polarized macrophages. M1 macrophages express typical Th1 cell-attracting chemokines such as CXCL9 and CXCL10, while M2 macrophages express the chemokines CCL17, CCL22 and CCL24 (Mantovani 2008; Martinez et al. 2006; Medzhitov and Horng 2009). Chemokines themselves can also influence macrophage polarization such as CCL2 promoting an M2-like phenotype while CXCL4 inducing a unique macrophage phenotype with a mixture of both M1 and M2 characteristics (Gleissner et al. 2010; Roca et al. 2009). Finally, cellular metabolism of iron, folate, and glucose is also differentially regulated between M1- and M2-polarized macrophages (Puig-Kroger et al. 2009; Recalcati et al. 2010; Rodriguez-Prados et al. 2010; Biswas and Mantovani 2012) (Fig. 2.1). In this regard, the expression of the protein metabolism enzyme, Transglutaminase 2 has been found to be a conserved M2 characteristics in human and mouse macrophages (Martinez et al. 2013). Finally, consistent with concept of polarization, the functional phenotypes expressed by macrophages in vivo or ex vivo in pathological conditions such as parasite infections, allergic reactions, and tumors reflect many aspects of polarized M1 and M2 macrophages [for review, (Sica and Mantovani 2012)]. However, macrophages with overlapping M1–M2 characteristics and shifts in their polarization states in course of pathological setting have also been noted suggesting the plasticity of these cells (Biswas and Mantovani 2010).

2.4 Transcriptional Regulation of Macrophage Polarization

Over the last few years, considerable progress has been made in toward characterizing the transcription factors, epigenetic mechanisms, and post-transcriptional events regulating macrophage polarization (Natoli and Lawrence); Also see Chapter 26 by Natoli and colleagues. IFN-γ was the first cytokine identified to induce M1 polarization (Nathan et al. 1983). Binding of IFN-γ to its receptor induces JAK 1/2-mediated phosphorylation and dimerization of Signal transducer and activator of transcription 1 (STAT1) (Shuai et al. 1993), which in turn engages responsive

elements in the promoters of M1 phenotype related genes such as iNOS, IL-12, and CXCL10 (Darnell et al. 1994). STATs are also involved in LPS-mediated M1 polarization through TLR4 activation. In response to LPS, the TRIF-dependent TLR4 pathway triggers IFN Regulatory Factor 3 (IRF3) activation which induces the expression of IFN-β. IFN-β in turn through the IFNAR triggers STAT1 and STAT2 phosphorylation. The STAT1/STAT2 heterodimer also recruits IFN-Recognition Factor 9 (IRF9) as part of a complex that binds the IFN-response gene elements (Stark et al. 1998). Another member of the IRF family, namely IRF5, whose stability is regulated through the interaction with the COP9 signalosome (Korczeniewska and Barnes 2012), was found to be activated in M1 macrophages. IRF5 activation in M1 cells regulates the expression of IL-12, IL-23, and TNF, thereby controlling Th1 and Th17 responses (Krausgruber et al. 2011). Expression of M1 macrophage-associated genes is also promoted by the Notch-RBP-J axis, which induces the synthesis of IRF8 by selectively augmenting IRAK2-dependent signaling via TLR4 (Xu et al. 2012). JNK activation is also required for pro-inflammatory macrophage activation and lack of JNK in macrophages protected mice against insulin resistance (Han et al. 2013).

While M1-promoting signals activate STAT1, IL-4 and IL-13 skew macrophages toward the M2 phenotype via STAT6 (Sica and Bronte 2007). Indeed, binding of IL4 or IL13 to their receptors promote the phosphorylation and dimerization of STAT6, which in turn recruits IRF4 and triggers the transcription of M2-associated genes, such as mannose receptor (*Mrc1*), resistin-like α (*Retnla*, *Fizz1*), chitinase 3-like 3 (*Chi3l3*, *Ym1*) (Gordon and Martinez 2010; Junttila et al. 2008) (Pauleau et al. 2004) as well as the inhibition of many inflammatory genes (Ohmori and Hamilton 1998). Another member of the same family, STAT3 is the main transcription factor regulated by IL-10 and induces the expression of several genes related to the M2-like phenotype (*Il10*, *Tgfb1*, *Mrc1*) (Lang et al. 2002; Gordon 2003; Mantovani et al. 2002). Moreover, among the M2-promoting transcription factors, STAT5 also finds a place due to its activation in response to IL-3 (Kuroda et al. 2009).

Members of the suppressor of cytokine signaling (SOCS) family block JAK/STAT pathway by negative feedback in macrophages. In fact, IL-4 and IL-13 up-regulate SOCS 1 and 2, thus inhibiting STAT1 pathway and interfering with M1 polarization. On the contrary, IFN-γ in concert with TLR stimulation, up-regulates SOCS3, which in turn inhibits STAT3, resulting in the dampening of M2 polarization (Spence et al. 2013; Whyte et al. 2011). A SOCS3-dependent pathway has also been found to be involved in RBP-J-mediated Notch signaling regulating macrophage polarization (Wang et al. 2010b).

IL-4-STAT6 pathway modulates various transcription factors promoting M2 polarization of macrophages. For instance, the nuclear receptor peroxisome proliferator-activated-γ (PPARγ) is constitutively expressed at low levels in macrophages but once induced by IL4-STAT6, it inhibits STAT, NF-κB, and AP-1, thus inhibiting M1 response (Odegaard et al. 2007; Ricote et al. 1998; Szanto et al. 2010). PPARγ activity similarly promotes a M2 phenotype in tissues and its expression is also induced by IL4-STAT6 pathway (Odegaard et al. 2008; Kang et al. 2008). Of relevance, STAT6 synergizes with Krüppel-like factor 4 (KLF4)

(Liao et al. 2011; Cao et al. 2010). Indeed, IL4 induces STAT6 phosphorylation to promote KLF4 gene expression. KLF4 in turn synergizes with STAT6 to promote M2 gene expression (*Arg-1*, *Mrc1*, *Fizz1*, *PPARg*) and inhibits M1 genes (*TNFa*, *Cox-2*, *CCL5*, *iNOS*) preventing NF-κB activation through the sequestration of the necessary co-activators. Thus, KLF4 functions as a point of no return in M1 versus M2 polarization: in the absence of KLF4 M1 polarization is facilitated and M2 polarization is impaired (Liao et al. 2011). In parallel to KLF4, KLF2 impairs macrophage activation by inhibiting the NF-κB/HIF-1α functions, even though the lack of KLF2 is not associated to defects in M2 marker expression (Mahabeleshwar et al. 2011).

Downstream of IL-4 signaling, human macrophages also activate c-Myc, which modulates the expression of genes associated with M2 activation (*Scarb1*, *Alox15*, and *Mrc1*), as well as STAT6 and PPARγ activation (Pello et al. 2012b). Accordingly, in an in vivo model, the myeloid-specific c-Myc deletion resulted in a delayed maturation of tumor-associated macrophages (TAMs) and a reduction of their protumoral functions (reduced expression of VEGF, MMP9, and HIF1α) that was associated with impaired tissue remodeling, angiogenesis, and reduced tumor growth (Pello et al. 2012a).

NF-κB is a key transcriptional regulator of both M1 and M2 polarization (also see Chapter 21 by Lawrence). LPS and TLR-induced NF-κB activation play a pivotal role in the expression of many inflammatory genes and orchestration of M1 polarization (Bonizzi and Karin 2004). Indeed, many M1 genes present a NF-κB binding site in their promoter region, including iNOS, CCL2, COX2, and CCL5 (Huang et al. 2009). In contrast, NF-κB activation also triggers a genetic program necessary for resolution of inflammation and M2 skewing of tumor-associated macrophages (Lawrence and Gilroy 2007; Hagemann et al. 2008). In this regard, nuclear accumulation of p50 NF-κB homodimers was observed in both TAMs and LPS-tolerant macrophages, suggesting a role of this transcriptional repressor in promoting M2 phenotype and impairing M1 polarization (Porta et al. 2009; Saccani et al. 2006). Thus, depending on the temporal framework, the stimuli involved and the relative amounts of different NF-κB homo- or heterodimers, this master transcription factor can drive macrophage polarization to distinct and contrasting outcomes. The nuclear receptor NR4A1 (Nur77) which is expressed in macrophages and within atherosclerotic lesions was recently found to inhibit the activation of the p65 NF-κB in macrophages, thus acting as a negative regulator of M1 polarization (Hanna et al. 2012).

Hypoxia Inducible Factors (HIFs) play important roles in macrophage polarization. HIF-1α and HIF-2α were found to be differentially expressed in M1- versus M2-polarized macrophages (Takeda et al. 2010). Moreover, Th1 cytokines promote HIF-1α activity via NF-κB and mediate transcription of iNOS (M1-associated gene); Th2 cytokines promote HIF-2α activation, which limits nitric oxide production by inducing arginase 1 (M2 associated gene) (Takeda et al. 2010). Earlier study with myeloid cell-specific HIF1α knockout also indicated its contribution to the inflammatory and bactericidal response of macrophages (Cramer et al. 2003). However, myeloid cell-specific HIF2α knockout has revealed its dominant role in macrophage inflammatory response to M1 stimuli and macrophage migration into

tumors (Imtiyaz et al. 2010). Further studies considering the temporal interaction of the HIF isoforms may shed further light on their differential contribution in macrophage activation and polarization.

2.5 Epigenetic Regulation of Macrophage Polarization

Epigenetic changes define modifications in DNA that do not alter the genetic sequence but regulate the expression of encoded information in a context-specific way. They usually occurs post-translationally and comprises modifications of histones, such as methylation, acetylation, and phosphorylation, that set the "histone code," aimed at controlling the "availability" of chromatin for selected transcription factors, thus regulating the final rate of the expression of a target gene (Ivashkiv 2012). Recent evidence suggests that macrophage polarization may be controlled by different chromatin states of relevant gene loci (Medzhitov and Horng 2009; Smale 2010; Natoli et al. 2011). As a general point of view, the gene loci involved in macrophage polarization may present three transcriptional states: (a) a repressed state, characterized by "repressive" histone marks, namely trimethylation of histone 3 lysine 9 (H3K9me3), lysine 27 (H3K27me3), and lysine 79 (H3K79me3); (b) an intermediate transcriptional state, characterized by the simultaneous presence of activating (H3K4me3, H3K9,14-Ac) and repressing histone marks (H3K9me3 and H3K27me3); and (c) an active transcriptional state, characterized by open chromatin configuration and active histone marks, such as trimethylation of histone 3 lysine 4 (H3K4me3) (Medzhitov and Horng 2009; Wei et al. 2009; Barski et al. 2007).

Epigenetic remodeling modulates macrophage activation, differentiation, and polarization. As an example, a reduced global DNA methylation is associated with myeloid commitment from hematopoietic stem cells, in comparison with that occurring during lymphoid commitment (Takeuch and Akira 2011). During macrophage differentiation, some pivotal transcription factors such as PU.1 and CCAAT/enhancer binding protein (C/EBP)α may directly bind and open the regulatory regions of several M1 genes induced in response to TLR ligands (e.g., TNF, IL-1β, IL-6, IL-12p40, CXCL10) (Ghisletti et al. 2010; Jin et al. 2011). During M1 macrophage polarization, IFNγ-induced STAT1 activation mediates chromatin opening (Chen and Ivashkiv 2010). In resting macrophages, inflammatory gene transcription is "silenced" by repressive elements, such as repressors (e.g., BCL6) or receptors that sequester activating factors (histone deacetylases and demethylases). TLR activation results in dissociation of repressors from relevant gene loci and in activation of demethylases, such as Jumonji JMJD3, JMJD2d, PHF2, and AOF1, that eliminate negative histone marks, thus making chromatin accessible to the transcription machinery (De Santa et al. 2009; Stender et al. 2012; Zhu et al. 2012). Among these enzymes, TLR engagement induces the expression of the H3K27 demethylases JMJD3 that is involved in fine-tuning the expression of a set of genes skewing macrophages toward M1 polarization (De Santa et al. 2009). However, in vivo evidence showed that JMJD3 is dispensable for M1 polarization, but is fundamental for M2 macrophage polarization to

helminth infection, through the induction of IRF4, required for M2 polarization, as mentioned before (Satoh et al. 2010). Noteworthy, M2 polarization in response to IL-4 seems to be independent of JMJD3, suggesting that macrophage polarization in one direction in response to distinct stimuli may follow distinct ways (Satoh et al. 2010). IL-4-induced M2 polarization is counteracted by HDAC3, which deacetylates enhancers of IL-4 responsive genes (Mullican et al. 2011).

Histone methylation is also controlled by oxygen availability. In macrophages, hypoxia impairs Jumonji histone demethylases activity, thus favoring the prevalence of repressive marks, (H3K9me2/me3), and the consequent inhibition of transcription of chemokine (Ccl2) and chemokine receptor (Ccr1 and Ccr5) genes (Tausendschon et al. 2011).

Epigenetic regulation consists of a further level of modulation of cellular functions and phenotypes. Thus, proteins that interact with post-translationally modified histones may represent an intriguing molecular target for new therapeutic strategies. To this regard, the bromodomain and extracellular domain (BET) family proteins recognize acetylated histones, thus promoting transcription by RNA polymerase II (Jang et al. 2005; Yang et al. 2005). A synthetic compound (I-BET) which inhibit the interaction between BET proteins and acetylated histones has been found to repress the expression of genes involved in M1 polarization in LPS-activated macrophages, thus providing a new potential tool against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis (Nicodeme et al. 2010). Compounds that inhibit JMJD3 and related demethylases were found to reduce the LPS-induced inflammatory cytokines production by human primary macrophages (Kruidenier et al. 2012).

Glucocorticoids (GCs) are one of the most potent anti-inflammatory drugs that interact with homodimeric nuclear receptors (GRs), which regulate transcription of target genes by binding to glucocorticoid response elements (GREs) (Glass and Saijo 2010). It was recently found that following TLR4 engagement by LPS in macrophages, GR cistrome is dramatically remodeled to an expanded inflammatory cistrone, that includes both GR-induced and -repressed genes (Uhlenhaut et al. 2013). Interestingly, negative GR enhancers selectively use the co-repressor GRIP1, interfere with the IRF3 activity, and present reduced histone acetylation, thus suggesting a role for the epigenetic regulation and chromatin status in driving the transcriptional effect on GR controlled target genes, beyond the GR itself (Uhlenhaut et al. 2013).

2.6 MicroRNA Regulation of Macrophage Activation and Polarization

Micro RNA (miRNAs) are small (20–22 nucleotides) non-coding RNAs which bind to the 3′ untranslated regions (UTRs) of target genes, thus repressing mRNA translation and/or inducing degradation of target gene transcripts, resulting in the inhibition of the target gene expression (Bartel 2009). A huge number of miRNAs have been identified and each miRNA may control several mRNA transcripts: this

post-transcriptional mechanism of gene expression regulation is emerging as a major player in modulating a number of biological processes.

An intense experimental effort has been made in identifying miRNAs that are differentially expressed in polarized macrophages and recent evidence describe a role for these non-coding sequences in regulating the differential gene expression profiles in macrophages during inflammation and tumorigenesis (Also see Chapter 28 by Locati et al.). In particular, both in human and murine monocytes and macrophages, TLRs signaling has been associated to the regulation of miRNAs, thus activating multiple targeting strategies that modulate expression of key molecules and fine-tune pro- and anti-inflammatory processes. The best characterized pro-inflammatory miRNA is miR-155, which is strongly induced by LPS or Type I interferons in both mouse and human monocytes and macrophages (O'Connell et al. 2007). The well-established pro-inflammatory function of this molecule is due to its ability in increasing the TNF transcript stability (Bala et al. 2011), promoting antiviral immunity through SOCS1 down-regulation (Wang et al. 2010a), and favoring atherosclerosis by targeting BCL6 (Nazari-Jahantigh et al. 2012). In contrast, miR-146a, the first miRNA shown to be induced by TLRs activation in macrophages, can itself regulate TLR signaling pathway by targeting key signaling molecules such as IRAK-1 and TRAF6 (Taganov et al. 2006), thus acting as negative regulator of inflammation (Jurkin et al. 2010) and in endotoxin tolerance (Nahid et al. 2009). TLRs engagement induces others miRNAs, namely, miR-9 (Bazzoni et al. 2009), miR-21 (Sheedy et al. 2010), and miR-147 (Liu et al. 2009), all of which have been demonstrated to operate a feedback control of the NF-κB-dependent response, by directly fine-tuning the expression of key members of the NF-κB family (miR-9) or down-regulating the translation of the pro-inflammatory tumor suppressor, programmed cell death 4—PDCD4, an inhibitor of IL-10 production (miR-21). MiR-125a/b play a dual role in the control of inflammatory circuitries, as they reduce the TNF transcript stability (Tili et al. 2007), but at the same time, also sustain inflammation enhancing NF-κB signaling by targeting the NF-κB negative regulator, TNFα Induced Protein 3 (TNFAIP3, A20) (Kim et al. 2012), and IRF4, thus resulting in an enhanced macrophage activation (Chaudhuri et al. 2011).

Considering their purpose in modulating TLR-mediated cell activation, anti-inflammatory stimuli may also act through miRNA induction or repression. To this regard, IL-10 has recently been show to directly induce miR-187 in TLR4-activated monocytes: miR-187 directly targets TNF mRNA and indirectly decreases IL-6 and IL-12p40 expression, through the down-modulation of IκBζ, a positive transcriptional regulator of these two cytokines (Rossato et al. 2012). In addition, our group recently identified miR-146b as a second IL-10-dependent miRNA and demonstrated its ability in dampening the production of inflammatory mediators by multiple targeting of components of the TLR signaling pathway (Curtale et al. 2013).

A clear role of miRNAs is emerging in macrophage polarization. To this regard, miR-125 and miR-29b interfere with M2 activation by targeting IRF4 and sustain M1 activation via targeting the NF-κB negative regulator A20, respectively (Chaudhuri et al. 2011; Graff et al. 2012). In the same direction, miR-155 targets

the IL-13 receptor α chain, thus interfering with M2 polarization, and C/EBPβ, a major transcription factor controlling the expression of M2 markers such as Arg1 and Chi3l3 (He et al. 2009), thus resulting in macrophage skewing toward the M1 phenotype.

In contrast, two intronic miRNAs are co-regulated together with their host M2 genes and thus their expression increases in response to alternative activation. MiR-378 is hosted in the first intron of the PPARγ gene and acts as a negative regulator as it targets the IL-4 signal transducer Akt1 (Ruckerl et al. 2012). MiR-511 is hosted in the fifth intron of the mannose receptor 1 gene (also known as CD206): it is highly expressed in M2 macrophages and TAMs and is shown to down-regulate the pro-tumoral genetic program of TAMs, inhibiting tumor growth (Squadrito et al. 2012). Quite recently, miRNA let-7c has been found to be involved in promoting murine M2 polarization, by targeting the negative regulator of TLR4-mediated inflammatory response C/EBP-δ and by regulating bactericidal and phagocytic activities of murine macrophages (Banerjee et al. 2013). Conversely, murine in vitro and in vivo evidence indicate that miR-19a-3p inhibits the M2 phenotype of macrophages, by targeting the protooncogene Fra-1, thus repressing its downstream genes VEGF, STAT3, and pSTAT3. As a result, miR-19a-3p was found to inhibit in vivo breast cancer progression and metastasis (Yang et al. 2013). Finally, a recent report identified a set of miRNAs specifically expressed in distinct subsets of M2 and M1 human monocyte-derived macrophages and showed their influence on cytokine profile (Graff et al. 2012). However, seven of the eight identified miRNAs were passenger strands, that usually are not included in the silencing complex, but are quickly degraded (Bartel 2004): the real functional significance for these miRNAs in macrophage polarization needs to be further clarified.

2.7 Concluding Remarks

Plasticity is a well-known characteristic of the monocyte–macrophage lineage. Within the tissue microenvironment, the complex integration of tissue-specific signals, microbial factors, and soluble mediators determines genetic re-programming, phenotypic changes, and differential activation of these cells. The pathologic consequences of macrophage polarization imply that specific targeting of polarized macrophage subsets (or activation states) can be considered as the final goal for therapeutic intervention. It is emerging that the therapeutic effect of some current drugs that were not specifically designed to target macrophages, such as PPARγ inhibitors, statins, zolendronic acid, and glucocorticoid hormones, are likely to act by targeting cells of the monocyte–macrophage lineage [for discussion see (Sica and Mantovani 2012)]. For instance, the clinically approved anticancer agent Trabectedin has recently been found to be effective because of its major effect in inducing depletion of TAMs (Germano et al. 2013). Further investigations of mechanisms and molecules involved in polarized activation of macrophages may

facilitate the finding of novel diagnostic and therapeutic tools aimed at modulating the multi-faced functions of mononuclear phagocytes.

While this book was in production, new transcriptomic studies on macrophages revealed a spectrum model of macrophage activation states extending the current M1 versus M2-polarization model (Xu et al. 2014). Simultaneously, an effort has been made by the community to suggest an uniform nomenclature for macrophage activation (Murray et al. 2014)

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Chapter 3
Alternative Activation of Macrophages: Concepts and Prospects

Siamon Gordon, Laura Helming, and Fernando O. Martinez Estrada

3.1 Background

As knowledge of macrophage receptors, sensors, phagocytosis, secretion, and antimicrobial activities was defined, it became apparent that macrophages in tissues within the healthy adult host could exist in different functional states; one basal, perhaps quiescent if not inert, the other partially or radically altered in response to endogenous and exogenous changes in their cellular and host environment (Gordon 2007). Macrophage activation was ill-defined, consisting of multiple changes in function elicited by diverse, non-specific infectious, and non-infectious stimuli. The studies by Mackaness and colleagues demonstrated that macrophages acquired an enhanced antigen-non-specific, but antigen-dependent antimicrobial activity (the "angry macrophage"), during infection by intracellular pathogens such as Mycobacteria, e.g., BCG and *Listeria monocytogenes* (Mackaness 1964). This depended on a CD4 T lymphocytes product, a lymphokine later identified as interferon gamma, enhancement of a respiratory burst and induced nitric oxide synthase able to generate potent antimicrobial metabolites such as oxygen radicals and nitric oxide. It turned out that natural killer cells also contributed to interferon gamma production, blurring the distinction between innate and adaptive activation of macrophages. Subsequent genetic studies in humans deficient in cytokines, receptors, or

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signaling of this "classical macrophage activation" pathway validated and extended its importance in cellular immunity to infection and inflammation; numerous studies have demonstrated the contribution of dysregulated pro-inflammatory cytokines such as TNF, proteolytic secretory products, and low molecular metabolites to tissue injury in immune and autoimmune disease (Gordon and Martinez 2010).

A parallel B lymphocyte, humoral pathway of host defense against extracellular pathogens depended on antigen-specific antibody production and the Fc and Complement receptors of macrophages and other myeloid cells, mediating phagocytic uptake and killing or extracellular lysis of microbes, as well as tissue damage.

The discovery of Toll-like receptors and their signaling pathways drew attention to the importance of innate pro-inflammatory mechanisms, with the macrophage a central participant, especially after "priming" by interferon gamma and immune complexes, thus linking the major arms of immunity. A search for direct, microbial-induced surface antigen markers and signaling of effector responses by macrophages yielded the Scavenger receptor MARCO, an adherence and phagocytic receptor, and Dectin-1, the fungal beta glucan receptor, as significant Innate recognition pathways.

The search for host protective mechanisms revealed potent anti-inflammatory cytokines such as IL-10 and TGF beta, antiproteinases, scavengers of oxidants, as well as inhibitory plasma membrane receptors, e.g., CD200, another innate regulatory molecule, and cytosolic inhibitors such as the SOCS proteins.

3.2 The Origins of the Alternative Activation Concept

Initial studies by Mokoena (Mokoena and Gordon 1985), Ezekowitz (Ezekowitz et al. 1986) and colleagues showed that Interferon gamma was a highly selective downregulator of expression of the lectin-like macrophage mannose receptor (MMR, CD206, MRC1), introduced to our laboratory by Stahl, who was interested in endocytosis of mannose-terminal ligands; these included beta-glucuronidase, recaptured after secretion and loss of the phosphate cap present on the family of lysosomal hydrolases (Shepherd et al. 1981). The subsequent discovery by Stein and others (Stein et al. 1992) that the cytokine IL-4, conversely, upregulated the MMR, led to the concept that IL-4, a Th2 cytokine could induce an alternative pathway of cell activation, and was not purely an inhibitory modulator of macrophage functions. The dichotomy of Th1 and Th2 CD4 T helper subsets had by then become widely accepted, with differential functions in intracellular infection of macrophages compared with extracellular parasitic infection and allergic diseases. We later extended this finding to IL-13 (Doyle et al. 1994), which shares a common receptor subunit with IL-4, as well as using its own receptor.

After a significant lag, the field gathered pace. Studies by De Baetselier (Raes et al. 2002), Allen (Loke et al. 2002), and their collaborators identified a signature of IL-4 induced genes in mouse alternatively activated macrophages (AAMs), including highly induced chitinase-like molecules (YM1/2), FIZZ-1, select chemokine

products (MDC, TARC), as well as the MMR. Brombacher and his colleagues (Herbert et al. 2004) confirmed the utility of this concept in parasitic infection and allergy models in mice, by deletion of the IL-4 receptor alpha-1 chain in macrophages. Studies by Paul and others established a major role in IL-4 signaling pathways (Paul 1997) for STAT6, now understood in some detail, as illustrated below.

A distinct Regulatory pathway of macrophage activation was described by Mosser and colleagues (Mosser and Edwards 2008), based on immune complex mediated FcR-dependent regulation of IL-12/IL-10 gene expression. Mantovani and colleagues (Sica and Mantovani 2012) put forward a classification of macrophage activation by Interferon gamma (M1) and distinguished three types of M2 activation, by IL-4/13, FcR, and IL-10. This terminology has been used widely and will be discussed further below. In this chapter we deal with IL-4/13 dependent AAM, except where noted otherwise.

A major difficulty in studying IL-4-dependent activation of macrophages in humans was the lack of correspondence of marker genes between mouse and human. While the MMR is useful, the prototypic mouse markers such as YM1/2 are not expressed in humans. In initial studies, Martinez (Martinez et al. 2006) demonstrated differences in chemokine and selected GPCR in IL-4 treated and control human monocyte-derived macrophages in culture. A detailed microarray and proteomic analysis by Martinez, Helming, and collaborators reported a signature of potential AAM markers for human gene expression, cellular and tissue analysis (Martinez et al. 2013). The enzyme transglutaminase-2 (TGM2), which is not restricted to macrophages, was proposed as a stable and conserved AAM marker, to be discussed further below.

3.3 The IL-4/IL-13 Pathway of Alternative Activation in Macrophages

3.3.1 Cytokine Sources

Apart from CD4 Th2 lymphocytes, type 2 innate lymphoid cells (ILC2), NKT and myeloid cells (basophils, mast cells, eosinophils) have been shown to express IL-4 and /or IL-13 message and protein, sometimes at very high levels (Spits et al. 2013). Recent studies by Hoyler (Hoyler et al. 2012), Mjosberg (Mjosberg et al. 2012), and their colleagues have shown that GATA-3, a TH2 regulator, controls ILC2 differentiation, maintenance, and function. After *Mycobacterium tuberculosis* infection of humans, tissue macrophages have been shown to express IL-4 message (Fenhalls et al. 2000), and infection by respiratory syncytial virus (RSV) (Shirey et al. 2010) and selected bacteria, e.g., *Francisella tularensis*, can induce an autocrine loop in macrophages involving IL-4 and its receptor (Shirey et al. 2008). Strober and his colleagues (Strober and Fuss 2011) have demonstrated the complex contribution of cytokines including NKT-cell derived IL-13 in the pathogenesis of inflammatory bowel disease, particularly ulcerative colitis.

Eosinophils in visceral adipose tissue have been implicated in the maintenance of AAM and metabolic homeostasis. ILC2 cells are the major source of IL-5 and IL-13, responsible for the accumulation of eosinophils and AAM after intestinal parasite infection. IL-33 promotes Th2 cytokine production by ILC2 (Molofsky et al. 2013).

3.3.2 Receptors and Signaling

IL-4 and IL-13 binding and signaling in macrophages lead to changes in the kinome, epigenome, transcriptome, and proteome of the cells and affect macrophage behavior and their response to the environment (Fig. 3.1). IL-4 and IL-13 are recognized by three receptors in human and mouse macrophages, but the system is older than that and sequence homologues of every receptor can be found already in Osteichthyes (bony fish) (Wang et al. 2011). The best-studied receptor complex is the IL-4 Type I receptor formed by the IL-4Rα1 and the common gamma chain (γ_c), reviewed in (Martinez et al. 2009). This receptor shows high affinity for IL-4. Extracellular binding motifs consisting of conserved paired cysteine residues and, in the membrane proximal region, a WSXWS motif mediates specificity of the complex. The IL-4 type II receptor arises when instead of the γ_c chain, IL-4Rα1 recruits IL-13Rα1. This dimer is the main functional receptor for IL-13, but is able to recognize both IL-4 and IL-13 with high affinity. Although signaling by IL-4 and IL-13 has been considered equivalent, there are differences between them in signal intensity and kinetics (Keegan et al. 1995). However, we have learned little about the differences between IL-4 and IL-13 in macrophages in past years. In addition to the signaling receptors, two additional receptors exist in the system, IL-13Rα2, which recognizes IL-13 with high affinity and a soluble form of IL-4Ra1. Both forms appear to play a role in mediating/regulating IL-4 effects, but the signaling cascade is not clear.

IL-4Rα1 or its binding partners lack endogenous kinase activity and require adaptor kinases to initiate signal transduction (Martinez et al. 2009). Signaling upon cytokine binding involves Type I or Type II receptor dimerization, recruitment of the Janus kinase family, the insulin receptor substrate family, and the phosphoinositide 3-kinase pathway. The accepted consensus is that Jak-1 associates with the IL-4Rα1 chain while Jak-3 associates with the γ_c chain, or Tyk-2 with IL-13Ra1. IRS1/2 becomes phosphorylated as a result of interaction with phosphorylated IL-4Rα1, presumably through the action of Jak1, Jak2, and Jak3 kinases and its phosphorylation drives interaction with the regulatory subunit of phosphoinositide-3-kinase (PI-3-K) and the adapter molecule, Grb-2. These interactions lead to the activation of the PI-3-K, Akt, and Ras/MAPK pathways. However, the Janus kinase mediated event best characterized is the phosphorylation of STAT6 (signal transducer and activator of transcription 6), which dimerizes, migrates to the nucleus, and binds to promoters of genes. Other transcription factors have been directly associated with IL-4 stimulation and a wider network is emerging which includes IRF4 and c-Myc (El Chartouni et al. 2010; Pello et al. 2012). The need for phosphorylation of many of these transcription factors remains to be defined. Other such as the PPR receptors

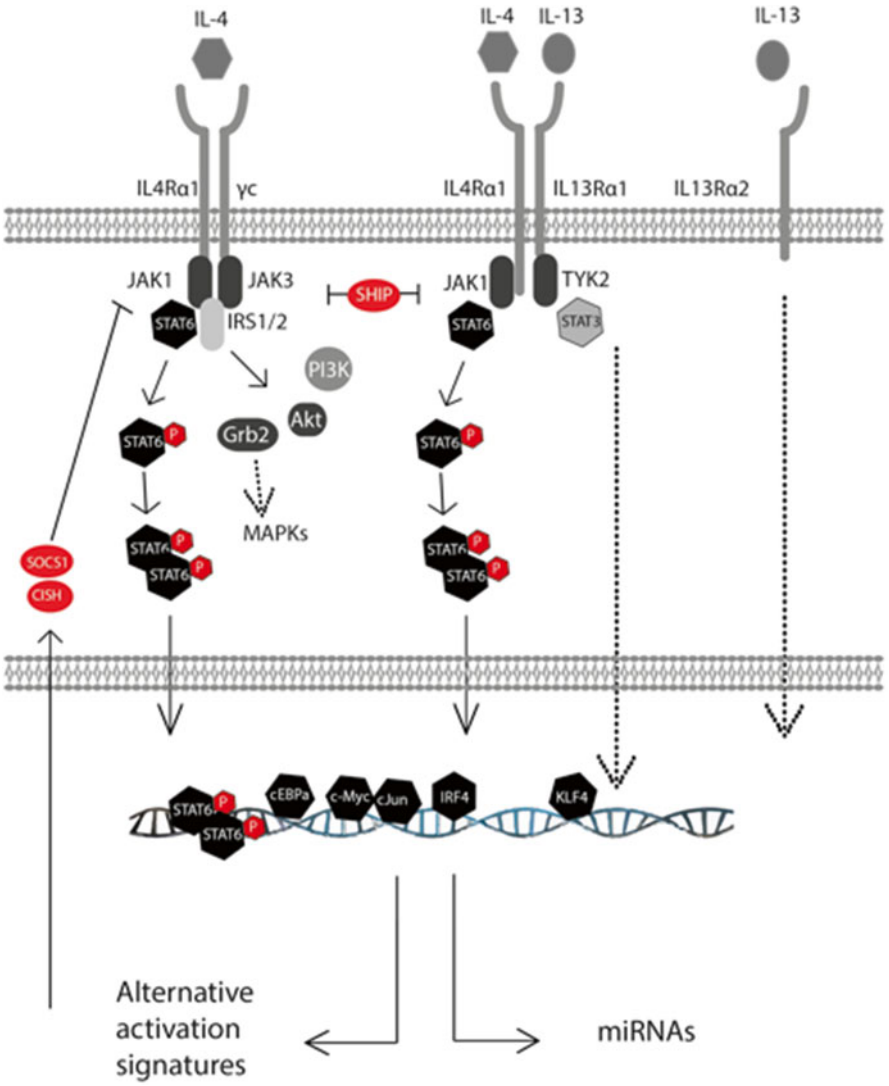

Fig. 3.1 IL-4 and IL-13 receptors and signaling. See text for details

has been shown to be important for IL-4 programmes, but indirectly. Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. Studies with PPARδ−/−, PPARγ−/−, and PPARδ/γ−/− macrophages reveal that both receptors are required for optimal expression of alternative activation markers but they are not amongst the genes regulated by the cytokine (Odegaard et al. 2007, 2008). IL-4 signaling is not only a positive process, and negative "brakes" are also required, e.g., transcription factors associated with Th1 responses such as T-bet are able to suppress an endogenous Th2-associated programme (Zhu et al. 2012).

The IL-4 induced signaling pathway has also negative modulators. SH2-containing phosphatases, SHP-1 and SHP-2, and the SH2-containing inositol-5-phosphatase (SHIP) are critical modulators of IL-4 signaling, and inhibition of phosphatase activity can result in Jak1 and Stat-6 activation (Rauh et al. 2004). Other SH2-domain modulatory proteins whose expression is induced in response to IL-4 are SOCS1 (for suppressors of cytokine signaling) and CISH (for cytokine-induced SH2), both upregulated within 1 h of IL-4 stimulation in human and mouse macrophages (Martinez et al. 2009). SOCS3 has been described to be an important modulator in murine bone marrow derived macrophages. miRNAs have also been associated with IL-4 signaling and these seem to act by controlling IL-4 receptor expression. MiR-7a-1, miR-155, miR-338-3p, miR-149, miR-10b, and miR-763 are some of a growing list of miRNAs induced and controlling IL-4 and IL-13 effects (Cai et al. 2012; Ruckerl et al. 2012).

Overall, a note of caution has to be made about what we know about the signaling system, i.e., the majority of existing evidence derives from studies on murine, chicken, and human cell lines, and less is known of what happens in primary cells. Thus, as for other aspects of alternative activation, further work is required to clarify the differences among species and models.

3.3.3 Gene Expression

Exposure to IL-4 induces a complex, consistent signature of upregulated, downregulated, and unchanged gene expression in macrophages, depending on the source, differentiation, and concomitant stimulation by infectious and other agents. Table 3.1 lists a comparison of selected stable human and mouse genes that are

Table 3.1 Selected IL-4 regulated markers in human and mouse macrophages (Martinez et al. 2006, 2013)

Gene symbol	Definition	Human	Mouse
MRC1	Mannose receptor, C type 1	X	X
TGM2	Transglutaminase 2	X	X
CD209	CD209 molecule	X	
CLEC10A	C-type lectin domain family 10, member A	X	
HOMER2	Homer homolog 2	X	
FCER2	Fc fragment of IgE, low affinity II, receptor for (CD23)	X	–
CD200R1	CD200 receptor	X	–
CCL22	Chemokine (C–C motif) ligand 22	X	–
SOCS1	Suppressor of cytokine signaling 1	X	X
CISH	Cytokine inducible SH2-containing protein	X	X
CHI3L3	Chitinase 3-like 3	–	X
RETNLA	Resistin-like alpha	–	X
ARG1	Arginase 1	–	X
ANXA4	Annexin A4	X	X
TRFC	Transferrin receptor	–	X

upregulated by IL-4; further details are provided in the report by Martinez, Helming, and others (Martinez et al. 2013). The differences between IL-4 and IL-13 at whole transcriptome level remain ill-defined, and further studies are needed to unveil them.

The regulation of genes by IL-4 not only requires modification of the transcription factor pool in the macrophages, but involves deeper epigenetic changes. The involvement of key epigenetics players by IL-4 such as Jmjd3, a histone H3 K27 demethylase is the clearest of examples at least in the mouse system (Satoh et al. 2010). Lawrence and Natoli (Lawrence and Natoli 2011) have reviewed aspects of transcription and chromatin remodeling in AAM. The fact that epigenetic mechanisms are involved adds an interesting regulatory level, which will be crucial to clarify in the context of the Macrophage Plasticity concept. Further details are definitively needed to elucidate how much of the transcriptional programmes regulated by IL-4 will be reversible in view of these epigenetic changes.

3.4 Selected Functions of AAMs

The role of AAM in parasitic infection and allergy has been well documented [(Allen and Maizels 2011), for example]. For a review of non-canonical functions such as the response to thermal stress, metabolic regulation and promotion of cognitive functions by AAM, see the perspective by Karp and Murray (2012), and for a recent comprehensive review on the role of IL-4 and IL-13 activated macrophages in infection, metabolic homeostasis and disease, see Van Dyken and Locksley (2013). In this chapter we draw attention to recent, less known and more controversial aspects in this field.

3.5 Endocytosis

Early studies by Montaner et al. (1999) demonstrated the potent effects of IL-4 and IL-13 on fluid phase as well as receptor mediated endocytosis. Membrane flow and delivery of ligands to lysosomes were markedly enhanced, in contrast with Interferon gamma and TNF. The MMR and transferrin receptors are both upregulated and may contribute to antigen processing and presentation, and iron homeostasis. Enhanced folate uptake may provide another essential nutrient.

Varin et al. (2010) provided further evidence of selective, profound effects on membrane dynamics of AAM (Varin and Gordon 2009). For example, phagocytosis of unopsonized *Neisseria meningitidis* by mouse peritoneal macrophages, mediated by Scavenger receptor A, was markedly reduced by IL-4 treatment. Unpublished studies by Milde and Helming have shown that the effects of IL-4 on phagocytic uptake depend on the particle, receptor, and whether the macrophage is single or part of a multinucleated, fused giant cell.

Balce et al. (2011) have reported that IL-4 increased cathepsins S and L, and that decreased NADPH-oxidase promoted a more reductive luminal environment, which further increased digestion within phagolysosomes.

3.6 Cell Proliferation

Allen and her colleagues (Jenkins et al. 2011) demonstrated that IL-4 promoted proliferation by resident alveolar macrophages in the lung as well as in other sites. Subsequent studies indicated that this was not a unique attribute of IL-4/13. Monocyte/macrophages elicited by inflammation also undergo some proliferation in response to IL-4, which is a weaker mitogen than M-CSF and GM-CSF.

3.7 Cell Fusion

Alternative activation of macrophages in vitro can induce cell–cell fusion and thereby the formation of multinucleated giant cells (Fig. 3.2). In vivo, giant cell formation is associated with chronic inflammation as well as the response to large foreign or poorly degradable materials. Multinucleated giant cells represent a histopathological hallmark of tuberculosis, schistosomiasis, and other granulomatous diseases (Helming and Gordon 2009). The role of Th2 cytokines in the formation of disease-associated giant cells is not completely clear, even though there are hints that alternative macrophage activation is involved at least for selected diseases (Gordon and Martinez 2010). The precise function and role of giant cells in disease

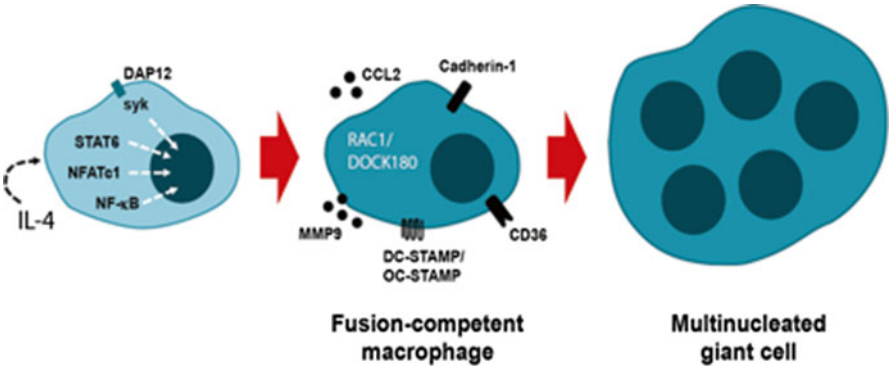

Fig. 3.2 Molecular mechanism of IL-4 induced macrophage fusion. A fusion-competent status is induced in macrophages via different signaling pathways, leading to increased expression of molecules involved in the fusion process: CCL2, DC-STAMP/OC-STAMP, Cadherin-1, and MMP9. In addition, IL-4 induced formation of multinucleated giant cells depends on cytoskeletal rearrangements (RAC1, DOCK180) and lipid recognition (CD36)

outcome is currently not known. Since they are often found close to large or poorly degradable material, it can be hypothesized that multinucleated giant cells display an enhanced degradative capacity by analogy to bone-resorbing osteoclasts which are also derived from fusion of macrophages.

Besides the IL-4/IL-13 stimulus, the macrophage fusion process depends on additional factors such as macrophage adhesion to fusogenic substrates as well as the differentiation/activation state of the macrophage (Helming and Gordon 2007). In contrast with macrophage fusion induced by alternative activation, the formation of osteoclasts is induced by receptor activator of NF-kB ligand (RANKL) and macrophage colony stimulating factor (M-CSF).

The molecular mechanism of macrophage fusion seems to be independent of the inducing stimulus. Most molecules implicated in macrophage fusion have been shown to be involved in both, IL-4- and RANKL/M-CSF-induced fusion (Helming and Gordon 2009). In order to fuse, macrophages first have to acquire a fusion-competent status by combination of different signals including the soluble cytokine stimulus and macrophage–macrophage interaction via surface receptors (e.g., TREM-2/DAP12). The respective intracellular signaling pathways required for fusogenic programming of macrophages involve NF-kB, NFATc1, STAT6, and Syk (Aguilar et al. 2013). The fusion-competent status is characterized by increased expression of CCL2, DC-STAMP/OC-STAMP, Cadherin-1, and MMP9, all shown to be involved in the fusion process. Furthermore, IL-4 induced macrophage fusion requires cytoskeletal rearrangements (RAC1, DOCK180) and proteinases (Aguilar et al. 2013). Besides the requirement for several known macrophage-associated molecules for fusion, the actual membrane fusion event is still poorly characterized. Changes in the membrane composition may be involved and changes in the lipid composition of the inner and outer membrane leaflet, i.e., exposure of phosphatidylserine could be detected during macrophage fusion. Intriguingly, CD36, one of the macrophage receptors recognizing phosphatidylserine was shown to be involved, not only in macrophage fusion, but also fusion of myoblasts (Helming and Gordon 2009; Park et al. 2012) pointing to a requirement for lipid recognition during cellular fusion events.

3.8 Transglutaminase 2

Transglutaminase 2 (Tgm2) is an AAM marker valid for both human and mouse macrophages. In a global microarray and proteomic analysis, Tgm2 represented the only molecule where upregulation by IL-4 was detected at both mRNA and protein level and which was upregulated in the majority of the macrophage in vitro models used (Martinez et al. 2006).

Tgm2 is a multifunctional protein, which can exhibit transamidating/deaminating, protein disulphide isomerase, GTP/ATP binding/hydrolyzing and even protein kinase activity as well as several non-enzymatic functions. It is associated with multiple functions including the cross-linking of extracellular matrix (ECM) proteins,

modulation of cell adhesion, activation of TGF-β and phospholipase A_2 (PLA_2), and a role in cell death/survival processes and autophagy (Gundemir et al. 2012). In macrophages, Tgm2 was shown to be involved in the phagocytosis of apoptotic cells and the release of pro-inflammatory cytokines after LPS stimulation (Szondy et al. 2003; Sarang et al. 2011). Both macrophage-associated features of Tgm2 are independent of its protein cross-linking activity, but rather dependent on guanine nucleotide binding. The precise role of Tgm2 in AAM is yet to be determined. Since Tgm2 is known to promote fibrosis, it may be involved in the pro-fibrotic activity of AAM.

3.9 Role of AAM in LPS Tolerance

The phenomenon of LPS tolerance, a refractory state in responses of the host and macrophages to repeated challenge with LPS has been studied for many years. This is known to involve the NF-kB pathways of signaling, nuclear translocation, and gene expression. A seminal study by Porta and colleagues in endotoxin-tolerant macrophages implicated inhibitory homodimers of the p50 NF-kB subunit, which negatively regulated STAT1 phosphorylation (Porta et al. 2009). This affects the expression of inflammatory cytokines to secondary LPS challenge, consistent with inhibition of the MyD88 and TRIF-dependent pathways. The contribution of p50 was clarified using peritoneal macrophages from p50 KO mice, unable to undergo endotoxin tolerance-mediated suppression of TNF-α production. In addition to having impaired cytokine responses, endotoxin-tolerant macrophages acquire a unique gene expression profile upon secondary LPS challenge that includes prototypic IL-4 alternative activation markers in mice, such as IL-10, TGF-β, CCL2, CCL17, and CCL22. The role of p50 in IL-4 or IL-13 signaling was not clarified by Porta et al., but the authors showed that endotoxin-tolerant macrophages retain their responsiveness to IL-4. A complex overlap of macrophage signatures across different forms of activation is increasingly accepted, although full genome transcriptome analysis comparing endotoxin tolerance and IL-4 profiles remain unpublished. A logical conclusion to Porta's observations would be that endotoxin tolerance and alternative activation have similar genetic signatures, but different regulatory networks. However, the contribution of the canonical IL-4-Stat6 pathway and NFKB-p50 to expression of alternative activation markers in vivo remains to be fully investigated; in a p50 KO OVA induced asthma model, macrophages do not express Arg1 and the levels of IgE in the blood, or CCL22 and CCL17 in the BAL, are severely reduced (Porta et al. 2009).

Vogel et al. recently extended studies of endotoxin tolerance to include IL-4Rα(−/−) and STAT6(−/−) macrophages, which are unable to give rise to AAMs (Rajaiah et al. 2013). The authors compared the effect of LPS and IL-4 mediated alternative activation and found that endotoxin-tolerant macrophages and IL-4 AAMs exhibited differences in classical and alternative activation markers; for example, IL-4 fails to induce the prototypic inflammatory cytokines induced by LPS. Vogel found that both states exhibited marked differences in NF-kB binding and MAPK activation; IL-4 conditioned-LPS treated cells failed to upregulate

expression of p50/p50 homodimers and did not alter the pattern of NF-kB p65 or p50 binding induced by pre-stimulation with LPS. The authors showed major differences in expression of negative regulators of LPS signaling, involving the MAPKs, and also IRAK-M, SHIP1, I-kBa, RelB, p-STAT3, STAT3, and p-STAT6. Using KO mice for IL-4Ra, STAT6, and IL-13, the authors showed that the system is not required to establish endotoxin tolerance in macrophages in terms of pro-inflammatory cytokine production, but interestingly found that the expression of Arg1 and Mrc1, genes, shared between IL-4 alternative activation and endotoxin tolerance, were markedly reduced. This, together with Porta's observation of decreased alternative activation markers in nematode infection and asthma in vivo models in p50 KO mice, appears to be another example of crosstalk between p50 and IL-4 receptor signaling pathways requiring further investigation.

3.10 Selected Pathologies: Allergic Inflammation

The role of Th2 cytokines in inflammation has been well defined in infections such as Schistosomiasis (Murray and Wynn 2011) and in asthma (Locksley 2010), in mouse models and humans. Asthma provides a convenient disease for validation of novel markers such as TGM2, to characterize AAM in situ. However, the heterogeneity of monocytes and macrophages, as well as the contributions from other cells such as basophils, Dendritic cells, and CD4-Th cells give rise to exceedingly complex interactions. For example, recent studies by Egawa et al. (Egawa et al. 2013) reported interactions between inflammatory monocytes recruited to skin by allergen-specific IgE, and basophils, which promoted their differentiation into AAM and attenuated the local inflammatory process. Studies by Plantinga et al. (Plantinga et al. 2013), using a house dust mite allergen model, identified migratory $CD11b^+$ conventional Dendritic Cells as a principal subset inducing Th2 cell-mediated immunity in lymph nodes, whereas monocyte-derived DC orchestrated allergic inflammation in the lung. Alternatively activated DC have also been implicated in the regulation of $CD4^+$ T cell polarization by MacDonald and colleagues (Cook et al. 2012). In their in vitro and in vivo studies, IL-4 treatment induced multiple activation markers with a different expression pattern to that of macrophages. DC IL-4 R alpha and RELM alpha, also an AAM marker in the mouse, were important in both Th1 and Th2 settings.

The nature and duration of cytokine exposure plays an important role in the severity and extent of inflammation. Milner and colleagues (Milner et al. 2010) reported that sustained delivery of IL-4, but not IL-13, by minipump or in a transgene model, lead to substantial $YM1^+$ tissue macrophage accumulation, erythrophagocytosis and disruption of hematopoiesis. Finally, the Dinauer group (Zeng et al. 2013) uncovered an apoptotic neutrophil efferocytosis-induced, IL-4-dependent macrophage-NKT-cell circuit, which suppressed sterile inflammation in the mouse peritoneal cavity. Deficient NADPH-oxidase activity impaired NKT-cell activation and prolonged the inflammation.

3.11 Nervous System

Several recent reports have drawn attention to a possible role for IL-4 and possibly AAM in stress-induced homeostasis including thermoregulation/cold shock (Nguyen et al. 2011), cognitive function impairment (Derecki et al. 2011), and inflammation and recovery following experimental spinal cord traumatic injury (Shechter et al. 2013). The thermal stress experiments involve catecholamines and macrophages in brown fat, and provided evidence for an essential role of the IL-4 R alpha. The cognitive dysfunction could be attributed to meningeal T cells, as well as macrophages as a possible source of IL-4. This experiment did not report a control experiment with a macrophage-selective IL-4Rα1 deficient mouse and may also not have controlled for confounding variables such as cold stress [see (Karp and Murray 2012)]. The group of Michal Schwartz and her collaborators (Shechter et al. 2013) implicated the choroid plexus as the portal of recruitment for a subset of monocytes, migrating via the cerebrospinal fluid to the site of neuronal loss and inflammation, which acquire M2-like healing properties, leading to faster recovery of motor function. In vitro generated M2-activated macrophages have been delivered to enhance recovery in similar experiments. This intriguing hypothesis awaits further characterization of the phenotype of the M2-like macrophages, in particular whether this is mediated by IL-4, present in the CSF, and whether the IL-4 R pathway is essential. It does raise the possibility, as in the EAE studies by Mosser and colleague (Mosser and Edwards 2008), that a different type of alternatively activated, IL-10 producing macrophages is able to counteract or switch the pro-inflammatory macrophage phenotype associated with nervous system damage.

3.12 Tumor Associated Macrophages

Tumor associated macrophages (TAM), like tissue macrophages, are heterogeneous and responsive to their environment, a capacity exploited by tumors to escape immune regulation. Macrophages are not alone and other hematopoietic system cells also infiltrate tumors including neutrophils, mast cells, and myeloid derived suppressor cells. Using genetic manipulation or clodronate liposomes to deplete all macrophage populations (Zeisberger et al. 2006), it has been demonstrated that the recruitment of macrophages can enhance tumor metastatic potential via enhanced cell migration, invasion and intravasation, neo-implantation as well as the promotion of angiogenesis. However, the macrophage role in tumors depends on their activation stage and it has been shown that they can promote or deter tumor growth, metastasis, vascularization, and outcome.

Sica and colleagues initially showed that tumor associated macrophages from mouse and human display alternative activation-like signatures with defective production of IL-12, exacerbated IL-10 production, and lack of p50/p65 NF-kappa B

activation (Sica et al. 2000). Later reports, for example by Carucci et al. (Pettersen et al. 2011) show that cutaneous squamous cell carcinomas, the second most common human cancer, contain abundant $CD68^+$ and $CD163^+$ macrophages. Some of these macrophages coexpress IL-4 related markers such as CD209 and CCL18. By gene set enrichment analysis (GSEA), the authors also found an abundance of classically activated genes confirmed by in situ co-localization of CD163 and phosphorylated STAT1, IL-23p19, IL-12/IL-23p40, and CD127; a subset of TAMs were bi-activated, as $CD163^+$ cells expressed markers for both classical and alternative activation shown by triple-label immunofluorescence. These and other data indicate that TAM, like other tissue macrophages, are heterogeneous populations with diverse types of activation.

While debate may continue about the activation profiles of macrophages in different tumors, the role of IL-4/IL-13 and alternative activated programs in tumor macrophages is becoming clearer. Coussens et al. studied the contribution of CD4T cells to pulmonary metastasis of mammary carcinomas and found that CD4T cells are important for this process, by directing, through IL-4, IL-13, and IL-10 production, the phenotype of macrophages toward alternative activation. IL-4 in particular sustained the expression of EGF in these cells, in combination with M-CSF, and was vital for tumor growth. These AAMs expressed ARG1, unlike the TAM of CD4T cell-depleted RAG1 mice, which expressed elevated levels of TNF, IL-6, IL-12p40, and IL1b (DeNardo et al. 2009). Linde et al., on the other hand, investigated the contribution of macrophage polarization to tumor progression in a model of VEGF-A-induced skin carcinogenesis, where transfection of the human non-tumorigenic keratinocyte cell line HaCaT with murine VEGF-A leads to malignant tumor growth in vivo (Linde et al. 2012). The tumors in this model are extensively vascularized, invasive, and carry high numbers of AAMs. Macrophage depletion from tumor-bearing animals in this model resulted in reduced tumor growth, inhibition of invasion, decreased proliferation, and reduced angiogenesis. Linde et al. showed that VEGF-A was responsible for the increased macrophage infiltration, but failed to induce M2 polarization. Interestingly, they identified macrophages themselves as an IL-4 source in vivo, suggesting an IL-4 autocrine loop for alternative activation, as observed for some viral infections (Shirey et al. 2010).

A last interesting example, leaning toward the role of alternative activation products in tumor progression, comes from the work of De Palma et al. (Squadrito et al. 2012). The mannose receptor gene is one of the most conserved alternative activation markers in human and mouse, and also characterizes a population of TAM. Interestingly, the MRC1 gene contains information for the mannose receptor transcript, but also an intronic microRNA: miR-511-3p. Enforced expression of miR-511-3p in TAM tuned down the protumoral gene signature of $MRC1^+$ TAMs and inhibited tumor growth in a Lewis Lung carcinoma model. The signature regulated by miR-511-3p contains only a few of the conventional IL-4 alternative activation markers, excluding a simple negative feedback loop, but indicates a regulatory network independent of the cytokine and important for tumor growth support.

3.13 Conclusion

It has become clear that apart from classical Th2-driven diseases (parasitic infection, allergies), the present concept of Alternative activation of macrophages is in danger of being oversimplified. The signatures of gene expression observed are often partial or mixed, overlapping with other cytokine, receptor, and signaling pathways in a chimaeric, even combinatorial fashion. Furthermore, the phenotype of macrophages in tissues is dynamic and depends on cellular location within the body, their capacity for plasticity, and additional microbial/environmental interactions. Genetic and epigenetic individual differences are striking in human outbred populations, compared with inbred mouse strains. In order to understand the mechanisms involved, we feel that it is essential to restrict definitions of AAM to distinct receptor/signal/gene expression pathways such as those for IL-4/13, to provide plausible targets for therapeutic manipulation. In order to evaluate the relevance of AAM to non- canonical physiologic functions and disease, mediated by Th2 cytokines, it is necessary to investigate genetically defined mouse models, as well as human pathologies, to validate molecular and cellular observations made in vitro. Ultimately, it should prove possible to reprogramme the polarization phenotype from classical to alternative activation of macrophages, and vice versa, as necessary.

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Chapter 4
Regulatory Macrophages and the Maintenance of Homeostasis

David M. Mosser and C. Andrew Stewart

4.1 Introduction

Macrophages are a physiologically diverse collection of cells whose function can vary tremendously depending upon their anatomical location and the stimuli to which they are exposed. The plasticity of macrophages allows them to assume diametrically opposed physiologies and their ability to rapidly change their physiology in response to environmental cues permits these cells to be major mediators of homeostasis. On one hand macrophages can produce toxic mediators that can kill pathogens and promote immune responses. On the other, they can inhibit immune responses and orchestrate wound healing and tissue repair. The fact that macrophages with opposite physiologies can exist in an organism argues that a strategy to deplete macrophages would likely be ineffective, because it would deplete cells on both ends of the spectrum. In order to focus attention on the diverse roles of these cells, several investigators have attempted to assign names associated with given functional states, despite the fact that most acknowledge that these states can rapidly change. In fact, we once suggested that assigning a functional name to a rapidly changing population of macrophages was analogous to "assigning a color to a chameleon," only to discover that no fewer than two other groups had previously made similar analogies. The assigning of names to macrophage populations based on their major activity, limitations notwithstanding, allows investigators to identify these cells in tissue or to direct drugs to specific subpopulations of macrophages in

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order to modulate immune responses. In this chapter we consider "regulatory macrophages" to be a broad general category of macrophages that are grouped by their functional activity. These activities include mitigating inflammation, regulating immune responses, and perhaps participating in tissue regeneration. We do not imply that all regulatory macrophages are identical. Rather, we propose that phenotypically diverse regulatory macrophages arise in response to a variety of exogenous and endogenous stimuli. We propose that all these regulatory macrophages share a common regulatory gene expression program leading to a reduced production of inflammatory mediators, and increased production of immunoregulatory cytokines and growth factors. We propose that these cells are major mediators of homeostasis and that without regulatory macrophages normal immune/inflammatory responses would invariably progress to immunopathology. Finally, we suggest that a part of the standard response of macrophages to inflammatory stimuli is the gradual transition from an inflammatory to a regulatory macrophage. This implies that macrophages can regulate their own physiology.

4.2 Initial Observations on Regulatory Macrophages

The original studies that paved the way for our entry into the field of regulatory macrophages came from initial observations made by Fayyaz Sutterwala, then a MD/PhD student in the lab, who was studying the process of phagocytosis. He developed a series of particles that bound specifically to individual macrophage receptors, including the complement receptors, the mannose receptor, a scavenger receptor, and the Fcγ receptors. Binding of particles to macrophages induced little to no cytokines on their own, however when macrophages were stimulated with TLR ligands in the presence of particles that ligated Fcγ receptors, the stimulation resulted in a dramatic alteration in cytokine production. Macrophages stimulated in the presence of IgG-opsonized particles exhibited a dramatic decrease in the production of the inflammatory cytokine IL-12 (Sutterwala et al. 1997), and an increase in the production of the anti-inflammatory cytokine IL-10 (Sutterwala et al. 1998). These alterations in cytokine production were quite profound (Fig. 4.1). It struck us as surprising that the ligation of a single receptor class on these cells could influence them so profoundly and "reprogram" them to become anti-inflammatory. Studies by several other groups have demonstrated that in vitro exposure of macrophages to CSF-1 (Fleetwood et al. 2007), cAMP analogues (Wall et al. 2009), surfactants (Bersani et al. 2013), glucocorticoids (Goerdt and Orfanos 1999), prostaglandins (Strassmann et al. 1994; MacKenzie et al. 2013), and adenosine (Nemeth et al. 2005) all result in macrophages with lower inflammatory cytokine profiles and increases in IL-10, similar to regulatory macrophages.

The regulatory macrophages that were generated in vitro resembled some of the macrophages that had previously been described in various anatomical sites in the body. For example, macrophages isolated from the uterine decidua exhibit enhanced production of IL-10 in the absence of stimulation (Heikkinen et al. 2003)

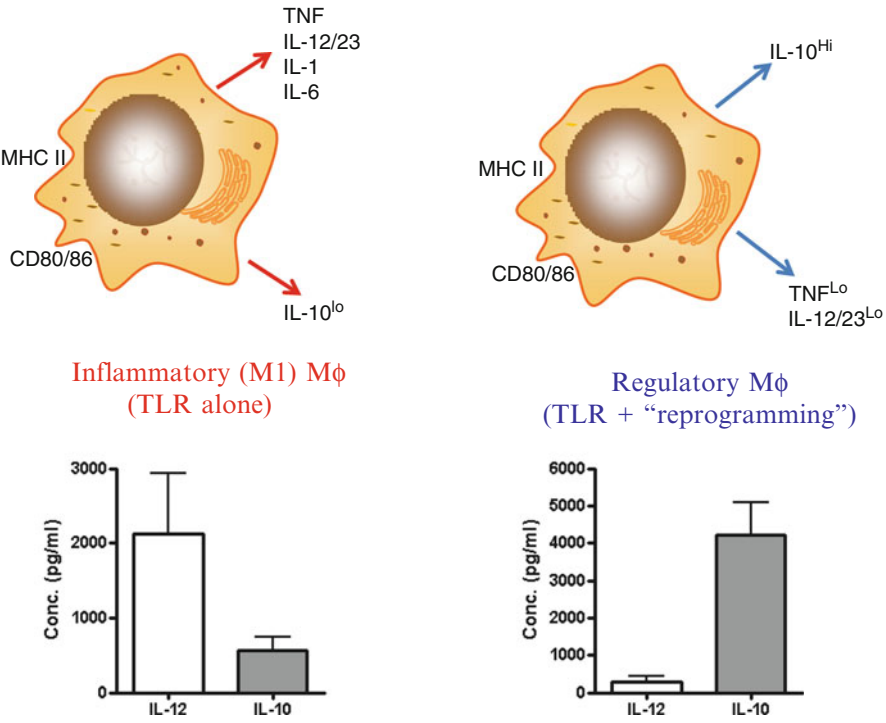

Fig. 4.1 Inflammatory (M1) and regulatory macrophages. Inflammatory M1 macrophages are generated by exposure to a TLR ligand. These cells express co-stimulatory molecules, produce high levels of inflammatory cytokines, but modest levels of anti-inflammatory IL-10. Regulatory macrophages secrete reduced amounts of IL-12 but overproduce IL-10. *Bottom*: IL-10 and IL-12 production by M1 macrophages (*left*) and regulatory macrophages (*right*). Data were obtained from ten independent experiments experiments (mean ± SEM)

and dampened inflammatory responses (Erlebacher 2013). These cells appear to play a critical role not only in promoting the tolerance of the semi-allogeneic fetus, but also in promoting postpartum uterine remodeling (Shynlova et al. 2013). Monocyte-derived macrophages in the injured retina promote progenitor cell renewal and produce IL-10 and TGF-β and reduce inflammatory cytokine production in the eye (London et al. 2011). A similar population of anti-inflammatory macrophages contributes to the development of the newborn lung (Jones et al. 2013). Macrophages with potent immunoregulatory properties have been isolated from a variety of tumors (Lewis and Pollard 2006; Mantovani and Sica 2010). Following ex vivo stimulation, these cells produce substantially less inflammatory cytokines but greater amounts of IL-10. The presence of tumor-associated macrophages is conversely associated with prognosis because these anti-inflammatory cells are understood to promote angiogenesis, increase invasion and metastasis by remodeling tissue matrix, and contribute to the local immunosuppression in the tumor (Pollard 2009). There are several other instances in which inflammatory

macrophages (so-called M1) undergo a temporal transition into macrophages with a regulatory phenotype. For example, during sepsis, cell wall components and nucleic acids from bacteria drive a strong host inflammatory response. Paradoxically however, monocytes isolated several days to weeks after the resolution of sepsis exhibit an anti-inflammatory phenotype with substantially reduced production of TNF and IL-6, but increased production of IL-1Ra and IL-10 (Monneret et al. 2004; Munoz et al. 1991; Cavaillon and Adib-Conquy 2006). This phenomenon has been collectively referred to as endotoxin tolerance, and in a mouse model of endotoxin tolerance, pre-exposure of mice to low levels of LPS protected mice from lethal doses of endotoxin (Freudenberg and Galanos 1988). A similar macrophage transition from an inflammatory to regulatory phenotype is necessary to orchestrate appropriate wound healing (Mantovani et al. 2013). In response to tissue injury, inflammatory monocytes migrate to the site of injury where they encounter necrotic tissue and inflammatory cleavage products of the extracellular matrix to induce the accumulation of inflammatory M1 macrophages. This macrophage population must transition into a growth promoting tissue regenerating population to orchestrate the wound healing response. Disruption of the transition to a regulatory macrophage population can delay or disrupt wound healing. A similar transition has been described to occur during normal muscle regeneration following exercise (Tidball and Villalta 2010). Therefore we think that regulatory macrophages are quite plentiful, that their regulatory phenotype can be transient or stably expressed in tissue, and that these cells are important for the maintenance of homeostasis.

4.3 Signaling in Regulatory Macrophages

Most of the studies concerning the generation the generation of regulatory macrophages were performed using cells that were stimulated with TLR ligands in the presence of immune complexes (IC). Many of the signaling events that we observed in these IC-treated regulatory macrophages are common to regulatory macrophages induced by different signals, including adenosine and prostaglandin E. In all cases, the induction of regulatory macrophages requires two signals; one to activate the transcription factors needed for cytokine production and a second to impart the regulatory bias (Fig. 4.2). The first signal includes ligands of any of the Toll-like receptors as well as many endogenous "danger signals." The first signal can be common to all M1 macrophages. The second signal induces little to no detectable cytokine production on its own. In our hands, for example, the addition of immune complexes (IC) that were devoid of LPS contamination induced no detectable IL-12 or IL-10 and negligible amounts of TNF from macrophages. This reinforces the idea that phagocytosis, per se, is not a trigger for macrophage cytokine production. This observation led us to consider FcγR ligation as a "reprogramming" signal that had a dramatic effect on the cytokines that were ultimately produced in response to TLR ligation (Signal 1). There are myriad signaling pathways emanated from the FcγR

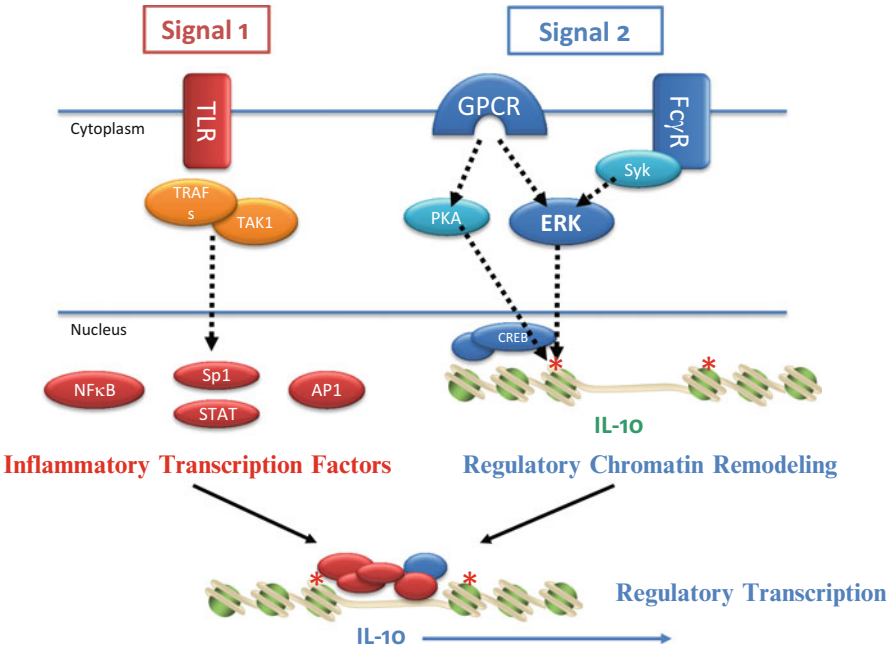

Fig. 4.2 Signaling in regulatory macrophages. Regulatory macrophages are generated by adding a "reprogramming" signal (*signal 2*) to macrophages stimulated with a TLR ligand (*signal 1*). These two signals cooperate to induce regulatory transcripts, including IL-10. Signal 1 activates the transcription factors necessary for cytokine induction. Signal 2 results in the phosphorylation of chromatin to make the *Il10* promoter more accessible to transcription factors

that influence cellular responses. One of the signaling pathways most responsible for reprogramming macrophage cytokine production was the activation of the mitogen activated protein kinase, ERK. FcγR ligation resulted in an activation of ERK and this activation was required for IL-10 production by regulatory macrophages (Lucas et al. 2005). The activation of ERK is not restricted to immune complex-stimulation of regulatory macrophages. Treatment of macrophages with prostaglandin E or adenosine also resulted in ERK activation, and in all cases macrophage reprogramming was ablated by inhibiting ERK activation (Edwards et al. 2009). We demonstrated that ERK activation had an indirect effect on the chromatin associated with the IL-10 promoter (Lucas et al. 2005). Histones associated with IL-10 became phosphorylated in an ERK-dependent manner, and these phosphorylation events made the IL-10 promoter more accessible to transcription factors (Zhang et al. 2006). Thus, we have proposed a "strength of signal" hypothesis to explain the development of regulatory macrophages, in which TLR ligation alone is insufficient to induce IL-10 production. Signaling from a second "reprogramming" signal is required to make the chromatin associated with the IL-10 promoter more accessible to the transcription factors that were activated by TLR ligation (Fig. 4.2).

There are several aspects of this reprogramming that are not well-understood. First, IL-10 is not the only gene that is induced in regulatory macrophages. There are over 100 genes that are significantly upregulated in macrophages stimulated with a combination of LPS + IC (regulatory macrophages) relative to LPS stimulation alone (M1). Many of the promoters associated with these induced genes have changes in histone phosphorylation following the reprogramming step (Zhang et al. 2006). It remains to be determined how these phosphorylation events are directed to these specific promoters and whether histone phosphorylation and chromatin remodeling is required for the induction of all the diverse regulatory transcripts. An important candidate for this reprogramming is the CREB transcription factor, a regulator of IL-10 expression whose activation is regulated by a variety of signaling pathways including ERK and cAMP-dependent protein kinase (PKA) that are induced by regulatory inducers (such as cAMP, PGE2, and Immune Complex) (MacKenzie et al. 2013) (Wall et al. 2009). Importantly, the access to regulatory transcripts via chromatin remodeling raises unexpected problems in developing reporter mice for regulatory macrophages. We have analyzed the induction of several of the genes that are markedly upregulated in regulatory macrophages in vitro using plasmid reporter constructs. IL-10 is a prime example. Macrophages transfected with a plasmid in which luciferase is driven by the *Il10* promoter upregulate luciferase in response to LPS alone but there is no additional luciferase expression in response to LPS + IC. This is despite the fact that this same population of cells expresses increased amounts of endogenous IL-10 response to the combined stimulation with LPS + IC. These observations suggest that the endogenous gene is regulated at the level of chromatin whereas the plasmid is not. This, of course has significant implications regarding the development of reporter mice that will allow the identification of regulatory macrophages. The second understudied aspect of regulatory macrophages is their functional activity in vivo. The induction of IL-10 in regulatory macrophages has been well-documented, and the role of IL-10 in modulating immune responses is well established. However, there are many genes that are induced in regulatory macrophages besides IL-10, and in fact many regulatory transcripts are induced in macrophages from IL-10-deficient mice. The biological significance of the other regulatory-associated transcripts has not been systematically studied. Regulatory macrophages overproduce growth and angiogenic factors as well as inducers of growth factors that may contribute to tissue regeneration, but the inability to specifically deplete regulatory macrophages from experimental animals has delayed our understanding of the myriad roles these cells may play in maintaining homeostasis in tissue.

4.4 Regulatory Macrophage Biomarkers

Given the dramatic differences in the physiology of the different macrophage subpopulations, it would seem logical that the depletion of one population could have a dramatic effect on immune/inflammatory responses. We hypothesize that in normal tissue, homeostasis is maintained by a balance between inflammatory (M1) macrophages and anti-inflammatory regulatory macrophages. We therefore

hypothesize that inducing regulatory macrophages may represent a strategy to prevent or reverse autoimmune pathology. Conversely, depleting these cells may improve vaccines or render chemotherapy more effective. A strategy to induce regulatory macrophages depends on identifying similarities among the different regulatory-inducing stimuli, and the induction of ERK seems to represent one key step in this process. Compounds that bind to macrophages and stimulate ERK may represent candidates for the induction of regulatory macrophages. The depletion of regulatory macrophages depends on the identification of reliable biomarkers that are expressed on regulatory macrophages but not on M1 macrophages. This would allow the targeting of cytotoxic drugs or antibodies to these cells. We have undertaken studies to identify regulatory transcripts with the eventual goal of exploiting these biomarkers to mark and target regulatory macrophages. There are upwards of 100 upregulated genes that are common to three different populations of regulatory macrophages. The level of induction of some of these transcripts is quite impressive and suggests that these gene products may both confer functional significance to regulatory macrophages and they may represent potential biomarkers to allow the identification of regulatory macrophages in tissue. Further studies are needed to identify stable reliable biomarkers for regulatory macrophages.

4.5 Intrinsic Development of Regulatory Macrophages

Above we describe several scenarios (sepsis, wound healing, and muscle development) in which macrophages transition from an inflammatory (M1) phenotype to a regulatory regenerative type. We hypothesize that the plasticity of these cells allows them to undergo this transition, and we propose that the switch from an inflammatory to a regulatory activation state is dependent on a reprogramming event similar to those described above. We recently identified a mechanism whereby TLR-stimulated macrophages control their own activation state and promote this transition to regulatory macrophages.

Stimulation of M1 macrophages by exposing them to TLR ligands results in an increase in glycolysis (Kellett 1966), leading to the generation of intracellular ATP (Cramer et al. 2003; Rodriguez-Prados et al. 2010). Macrophages release a portion of this ATP into the extracellular milieu via pannexin-1 channels (Cohen et al. 2013). Extracellular ATP (eATP) is rapidly hydrolyzed to adenosine by the coordinated efforts of two ectoenzymes expressed on the surface of macrophages, CD39 (nucleoside triphosphate diphosphohydrolase) and CD73 (5′ nucleotidase) (Fig. 4.3). Within hours of stimulation, macrophages efficiently convert released eATP into adenosine. Adenosine is a “reprogramming” signal that induces the production of regulatory transcripts in macrophages. We also demonstrate that following TLR ligation, macrophages selectively increase their expression of the adenosine 2b receptor (A2bR), thereby enhancing their sensitivity to adenosine. Collectively, these observations suggest that an integral part of the response of macrophages to TLR ligation is the production of ATP and its conversion to adenosine, which binds to the A2bR and induces the transition into regulatory macrophages.

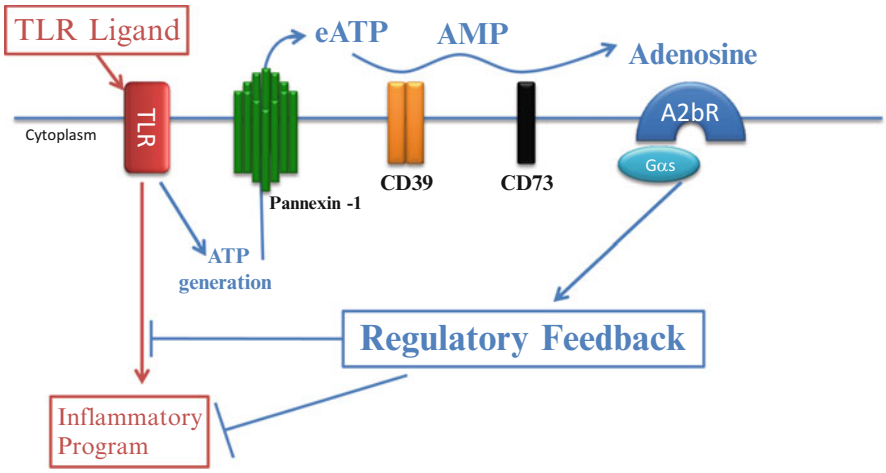

Fig. 4.3 Adenosine-mediated autocrine regulatory feedback in macrophages. TLR-stimulated macrophages increase glycolysis and produce ATP. ATP is secreted via pannexin-1 channels. ATP is sequentially hydrolyzed by CD39 and CD73 to adenosine. Adenosine binds to A2b receptors to "reprogram" macrophages and produce regulatory transcripts

Therefore, this work reveals a novel negative feedback mechanism controlling macrophage activation responses that is dependent on the macrophage's ability to convert eATP into immunosuppressive adenosine. Interestingly, the A2bR is a G-protein coupled receptor that associates with Gαs, therefore we hypothesize that the generation of cAMP plays an important role in mediating this effect. We previously demonstrated that treating macrophages with cAMP could promote the development of regulatory macrophages (Edwards et al. 2009), and therefore hypothesize that adenosine signaling through the A2bR may similarly influence macrophage physiology.

Our data suggested that the inflammatory response of macrophages to TLR ligands is normally transient, and this was confirmed experimentally. In the presence of CD39 blockade, macrophages release higher levels TNFα and IL-12 and this production is sustained over time, even after removal of the stimulus. To demonstrate the biological relevance of these observations, we examined a mouse model of septic shock and demonstrated that the transfer of only a million CD39-deficient macrophages into wild-type mice challenged with a sub-lethal dose of endotoxin resulted in endotoxin lethality. Furthermore, macrophages isolated from mice receiving wild-type macrophages exhibited elevated levels of regulatory transcripts, including IL-10, Arg1, HB-EGF, and IL-33 and low levels of TNFα and IL-12. This observation supports the theory that the development of regulatory macrophages is associated with decreased inflammation and increased survival of the host.

Based on our recent observations, we propose a new model of macrophage activation that places CD39 as a key molecular control used by macrophages to toggle between inflammatory and regulatory states (Fig. 4.3). This mechanism suggests that macrophages, themselves, are capable of controlling their own activation state during inflammation. We further propose that the development of macrophage-specific therapeutics targeting the synthesis, release, and hydrolysis of ATP by macrophages may greatly broaden treatment options for a variety of inflammatory diseases.

4.6 Summary

Macrophages have been associated with both the progression and the resolution of inflammation. An association with such diverse processes is due to the remarkable plasticity of these cells. Here we propose that regulatory macrophages are important in the resolution of inflammation, and represent a relatively understudied population of cells that can be exploited to manipulate immune responses. Their induction can lead to immunosuppression whereas their transient depletion can enhance immune responses. A better appreciation of the complexity of macrophages and their plasticity has begun to develop, and we are optimistic that this will lead to the development of new therapeutics directed at macrophages. The initial discovery of alternatively activated macrophages (Stein et al. 1992), also called M2a (Mantovani et al. 2013) described earlier in this book, expanded our appreciation of the diversity of macrophages and demonstrated that not all activated macrophages are the same. The regulatory macrophages that we describe in this chapter are not generated in response to the T_H2 cytokines IL-4 or IL-13 nor do they depend on Stat 6 for their activation (Edwards et al. 2006). Thus they are quite distinct from alternatively activated macrophages, and efforts to modulate immune responses with IL-4 or IL-13 will not yield results similar to those described in this chapter. The demonstration that regulatory macrophages represent a population of cells that is quite distinct from both alternatively activated and classically activated macrophages represents another step in the direction of developing subset-specific therapeutics to manipulate immune responses at the level of macrophages. We predict that these cells will express panels of biomarkers that are unique to regulatory macrophages to allow for their identification in tissue and their depletion while sparing inflammatory M1 and alternatively activated macrophages.

Although regulatory macrophages were only recently characterized, we propose that they are quite plentiful in tissue and that many different tissue-specific reprogramming signals can give rise to populations of macrophages with immunoregulatory characteristics. Finally, we predict that inflammatory M1 macrophages will normally transition into anti-inflammatory regulatory macrophages by producing an endogenous reprogramming process that involves the hydrolysis of ATP to adenosine. The reprogramming of macrophages by adenosine is similar to reprogramming by cAMP, PGE_2, or immune complexes. We propose that each of these populations of macrophages shares a core of common transcripts that justifies grouping them together into a single regulatory category. We suggest that it is more productive to stress the commonality between these regulatory cells because their depletion would be predicted to enhance immunity, whereas their induction would lead to a mitigation of inflammation.

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Part II
Macrophages and Their Diverse Functions

Chapter 5
Phagocytosis

Andrea J. Wolf and David M. Underhill

5.1 Introduction

Phagocytosis is the process by which cells engulf large (typically ≥0.5 μm) particles, microbes, cellular debris, or portions of the extracellular environment. Phagocytosis, literally "cell eating", was originally described by Ilya Mechnikov over a hundred years ago, for which he received the Nobel prize in 1908 together with Paul Ehrlich. During phagocytosis, a cell engulfs a target with its plasma membrane to form a new intracellular compartment called the phagosome, which acidifies and fills with proteolytic enzymes to degrade or kill the target. The origins of phagocytosis can be traced back to requirements for nutrient acquisition in single-celled organisms such as amoebae. These single-celled organisms internalized and degraded other microbes in order to obtain the basic building blocks of life: carbohydrates, lipids, and amino acids. As multicellular organisms evolved specific organs to compartmentalize nutrient degradation, phagocytosis of particulate matter by individual cells became restricted largely (but not exclusively) to specialized cells within the immune system including macrophages, dendritic cells, and neutrophils. These phagocytes use the process of phagocytosis to survey their microenvironments for danger and to kill potentially harmful microbes. It is also necessary for clearance of apoptotic cells and tissue debris as part of normal tissue homeostasis and repair.

Information collected during phagocytosis through the engagement of membrane receptors and activation of intracellular sensors determines the degree and intensity of the immune response, cell recruitment, inflammation, and tissue repair.

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When phagocytes engage microbes, they produce a host of cytokines and chemokines that define the local and systemic responses to the threat. While many studies have documented the abilities of immune cells to detect microbial products, these studies most commonly have focused on elaborating mechanisms of detection of soluble molecules shed from microbes. A prime example is the detection of lipopolysaccharides by Toll-like receptor 4 (TLR4). However, the process of phagocytosis itself is an integral part of how immune cells engage and respond to microbes, and recent data from multiple labs have demonstrated that internalization and degradation of microbes significantly alters the degree and composition of the inflammatory signals induced compared to surface receptor engagement alone. Following killing and degradation of microbes, phagocytes play a specialized role in presenting antigens derived from the microbes to the rest of the immune system. Antigen presentation, together with cytokines induced during phagocytosis and sensing, orchestrates the subsequent adaptive immune response, T cell activation, and polarization. This chapter will try to integrate our current understanding of the receptors and mechanisms used by phagocytes to internalize particles with our understanding of how the cells use the process to gather information that contributes to inflammation and the overall immune response.

5.2 Diversity in Mechanisms of Phagocytosis

Phagocytosis and the related process of macropinocytosis are actin-mediated forms of internalization. While phagocytosis tends to be restricted to specialized phagocytic cells, almost all cells in the body have the ability to internalize soluble molecules and some extracellular fluid through the non-actin associated process of endocytosis. Since phagocytosis is simply the process of a cell engulfing a particle, it is tempting to imagine that the process is the same regardless of the particle being ingested. In fact, over the last several decades it has become clear that the molecular and cellular mechanisms of phagocytosis can vary substantially depending on the type of particle and the cellular receptors engaged (Table 5.1). In an attempt to devise categories describing the different processes that have been observed, investigators often talk about "zippering phagocytosis," "triggered phagocytosis," and "sinking phagocytosis (Fig. 5.1)." These descriptions have been with us since at least the work of Silverstein and colleagues and Kaplan in the 1970s (Griffin and Silverstein 1974; Kaplan 1977; Griffin et al. 1975, 1976). To these we should add autophagy, the process by which cells engulf cytoplasmic material, including sometimes microbes, within intracellular membranes.

5.2.1 Zippering Phagocytosis

The zippering model of phagocytosis (sometimes also described as "reaching phagocytosis") is characterized by sequential receptor engagement that progressively draws the plasma membrane outwards around the particle to fully surround

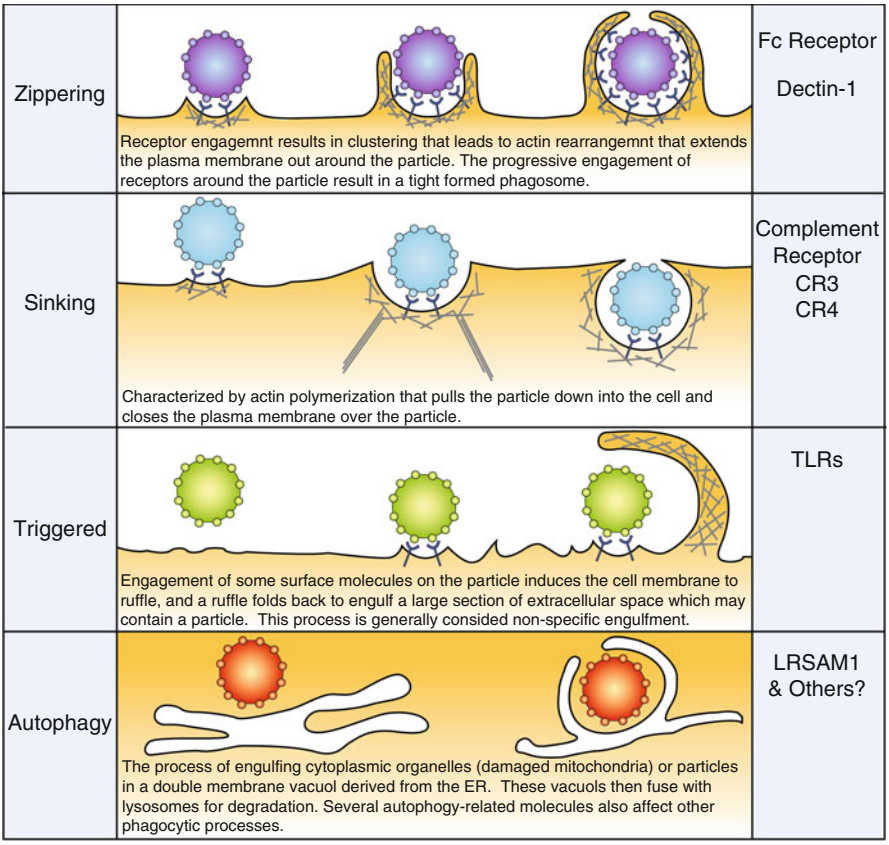

Fig. 5.1 Types of phagocytosis. The panels summarize the sequences of events associated with various types of phagocytosis and note examples of receptors involved with these processes

it and then pull it towards the cell body. Well-studied examples of zippering phagocytosis include Fc receptor engagement by antibody-opsonized particles as well as Dectin-1 binding to fungal cell wall β-glucans. Fc receptor engagement induces receptor clustering at the site of particle contact and phosphorylation of dual tyrosine residues in the receptors' intracellular "Immunoreceptor Tyrosine-based Activation Motifs" (ITAMs) (Abram and Lowell 2007; Goodridge et al. 2012). These dual-phosphorylated tyrosines facilitate binding to dual SH2 domains of the tyrosine kinase Syk and activating the kinase for downstream signaling including completion of phagocytosis and activation of the NADPH oxidase, the enzyme responsible for production of antimicrobial reactive oxygen species. Signaling by Dectin-1 is similar, although it's "HemITAM" motif includes only one tyrosine, and while Syk activation is required for NADPH oxidase activation, it is dispensable for phagocytosis (Rogers et al. 2005; Herre et al. 2004; Underhill et al. 2005). For both receptors, downstream activation of phosphatidylinositol-3-kinase (PI3K) is not necessary for initial actin polymerization at the site of particle contact,

Table 5.1 Representative phagocytic receptors and their ligands

Receptors	Ligands	Type of phagocytosis
Fc receptor		
FcγRI (CD64)	IgG-opsonized particles IgA-opsonized particles	Zippering
FcγRII (CD32)		
FcγRIII (CD16)		
FcαRI (CD89)		
Complement receptor		
CR1 (CD35)	MBL, C1q, C4b, or C3b opsonized particles	Sinking
CR3 ($\alpha_M\beta_2$, CD11b/CD18)	C3b-opsonized particles	
CR4 ($\alpha_x\beta_2$, CD11c/CD18)	C3b-opsonized particles	
Other integrins		
$\alpha_5\beta_1$	Fibronectin/vitronectin opsonized particles	Unclear
$\alpha_4\beta_1$		
$\alpha_v\beta_3$		
Scavenger receptors		
SRA	Bacterial cell wall components	Unclear
SRB		
MARCO		
Others		
Dectin-1	β1,3-glucan	Zippering
Mannose receptor	Mannan	Zippering
CD14	LPS	Triggered (non-specific)
C1qR	C1q	Unclear
TLRs	LPS, lipoproteins	Triggered (non-specific)

but experiments with inhibitors suggest that it is required for efficient membrane extension and particle internalization (Araki et al. 1996; Cox et al. 1999). In addition, phospholipases are activated to drive formation of diacylglycerol (DAG) and IP3 which induce a classic Ca^{2+} signaling cascade. There has been significant disagreement regarding the importance of Ca^{2+} signaling for phagocytosis; it may be important for some individual Fc receptors, but the combinations of receptors engaged by most particles appear to make Ca^{2+} signaling nonessential for internalization. However, Ca^{2+} signaling may be more important for dissolution of the actin network, proper ROS production, and phagosome maturation (Nunes and Demaurex 2010). Also essential to the zippering model is the activation of the Rho GTPases Rac1 and Cdc42 together with GTP exchange factor Vav, which are important for organizing actin polymerization at the clustered receptors and at the leading edge of the membrane as it extends around the particle (Swanson 2008). The importance of continuous and progressive receptor engagement and signaling during zippering phagocytosis was demonstrated by the elegant observation that opsonization of just one side of a bead results in the particle being only half-eaten (Griffin et al. 1976).

5.2.2 *Sinking Phagocytosis*

Phagocytosis of complement-opsonized particles is the main example of sinking phagocytosis. Unlike the zippering model, sinking phagocytosis does not involve prominent extensions of the plasma membrane out around the particle. Instead the particle is pulled into the cell and the plasma membrane closes in around it. Like zippering, sinking phagocytosis involves significant actin cytoskeletal rearrangement. Complement opsonization of particles can occur through three pathways. First, the classical pathway involves antibody bound to the surface of the microbe facilitating assembly of a C1 complex that promotes activation of a C3 convertase which drives C3b deposition. Variations on this scheme include microbe recognition by the membrane-associated lectin receptor SIGN-R1 that can associate with C1q to promote complement deposition (Kang et al. 2006) and direct recognition of certain microbes by C1b (Mueller-Ortiz et al. 2004). Second, the lectin pathway involves binding by soluble pattern recognition receptors such as collections (e.g., mannose binding lectin) or ficolins that directly organize assembly of the C3 convertase. Finally, the alternative complement activation pathway is initiated simply through binding of spontaneously activated C3 directly to microbial surfaces.

In all cases, the relevant endpoint is conversion of C3–C3b thus exposing a reactive thioester bond that can covalently link C3b to the surface of the microbe. Recognition of C3b is mediated by three families of receptors. CR1 and CR2 belong to a family of receptors containing short consensus repeat (SCR) sequences (also called complement control protein repeats (CCPs) or sushi domains) and are primarily involved in the removal of immune complexes and B cell activation respectively. While CR1 has been shown to mediate phagocytosis in neutrophils, tissue macrophages do not use CR1 for phagocytosis (Sengelov et al. 1994). CRIg is an immunoglobulin superfamily member receptor that has only recently been described on a limited subset of tissue macrophages throughout the body. While these two superfamilies of complement receptors primarily play a homeostatic role in the body, the recognition of complement-opsonized microbes is principally mediated by the β_2 integrin family members CR3 and CR4. Expressed on all types of phagocytes, CR3 and CR4 are heterodimers composed of the common β_2 integrin, CD18, together with either alpha subunit CD11b (α_M) or CD11c (α_X) respectively. These proteins play a role in cell interaction with extracellular matrix components as well as in microbe clearance. Patients lacking β_2 integrins have been diagnosed with leukocyte adhesion deficiency type 1 (LAD1) and present with recurring bacterial infections (Bunting et al. 2002; Hogg et al. 2002). Unlike the phagocytic receptors involved in zippering phagocytosis, the complement receptors are generally not constitutively active as phagocytic receptors. They require an additional signal, usually in the form of cytokines (e.g. TNFα), chemokines, or microbial products (e.g. LPS), in order to increase surface aggregation of the receptor and initiate activation of protein kinase C and the GTPases, Rap1, and RhoA (unlike the zippering receptors that require Rac1-mediated actin polymerization), shown to be involved in initiation of actin polymerization. Despite the involvement of these seemingly

inflammatory secondary signals, complement-mediated phagocytosis often does not involve additional inflammatory responses, and unlike Fc receptor-mediated phagocytosis does not typically trigger a reactive oxygen burst (Underhill and Ozinsky 2002).

5.2.3 Triggered Phagocytosis

The remaining category of phagocytosis of extracellular targets is triggered phagocytosis, also known as macropinocytosis or "cell drinking." Triggered phagocytosis is characterized by formation of large actin-rich ruffles on the cell membrane that merge to engulf indiscriminant portions of the extracellular milieu that can include particulate matter 0.5–5 μm in size nearby or bound to the cell surface (Weed and Parsons 2001). The membrane ruffles associated with triggered phagocytosis are induced by many different stimuli including ostensibly non-phagocytic receptors like TLRs and growth factor receptors. For example, dendritic cells and macrophages stimulated with LPS internalize large quantities of extracellular fluid containing antigens, and this is abolished in the presence of the actin polymerization inhibitor cytochalasin D (Poussin et al. 1998; West et al. 2004). In addition, *Salmonella typhimurium* contact with the surface of macrophages induces internalization via membrane ruffling that is dependent on the expression of the microbial virulence factor phoP, but appears to be independent of traditional phagocytic receptors (Alpuche-Aranda et al. 1994). As discussed more below, this category blurs the lines of what really defines a "phagocytic receptor." Can we think of a growth factor receptor as a "phagocytic receptor" when it does not bind to a microbe but it does promote internalization? Can we think of a TLR as a phagocytic receptor if it generally promotes internalization all over the ruffling membrane of a cell rather than just specifically at the site of particle contact?

5.2.4 Autophagy

Autophagy (literally "self-eating") is the process by which cells engulf, degrade, and recycle cytosolic material and damaged organelles (Billmann-Born et al. 2010). Although the term includes the processes of "micro-autophagy" and "chaperone-mediated autophagy," for our discussion here we are focused on the process of engulfing large volumes of cytosolic material that can include entire organelles such as mitochondria. While traditional phagocytosis involves mobilization of the plasma membrane to wrap around an extracellular target, autophagy involves mobilization of intracellular membranes to seal off an intracellular space. Unlike a traditional phagosome, the newly formed "autophagosome" is initially enclosed by a "double membrane" that results from the folding of intracellular membranes to form the compartment (Mizushima et al. 2002). The inner membrane is lost, and the compartment

then matures by fusion with common components of the endolysosomal pathway just like a traditional phagosome. Signals that direct autophagy to specific intracellular structures are an active area of research, and recent studies have suggested that ubiquitination of bacterial cell walls is important for targeting autophagy components to cytosolic bacteria (Knodler and Celli 2011). One recent study by Huett et al. has suggested LRSAM1, a cytosolic E3 ligase, as important for directly recognizing *Salmonella* cell walls via its leucine-rich repeat (LRR) domain and targeting the bacteria for autophagy (Huett et al. 2012).

5.2.5 Even More Diversity

It is tempting to assume that when a phagocyte uses a particular receptor to engage a particle and trigger phagocytosis the process will progress identically for all other like particles. However, not all phagosomes are equal. Even when examining uniform, carefully defined particles, phagosomes within the same cell can be significantly different (Griffiths 2004). Henry et al. observed that during phagocytosis of uniform model IgG particles by macrophages, some phagosomes rapidly accumulated phosphatidylinositol 3-phosphate (PI3P) and lost it within minutes, while others retained this key signaling molecule for hours (Henry et al. 2004). Phagosome variation likely starts from the moment of contact, with exclusion of certain plasma membrane receptors and lipids making room for particle binding and receptor clustering; every cell has hundreds of membrane proteins and diverse types of lipids, and these membrane components are not uniformly distributed. Add to this the fact that most biological particles have complex surfaces and likely engage varying numbers of several different types of receptors, and phagosomal variation quickly becomes a given. Still, the immunological significance of this heterogeneity remains to be understood. One possible avenue for exploration is the differing requirements for efficient killing of an organism and for efficient processing and presentation of suitable peptide antigens. In the former case, rapid destruction is the key, while in the latter, preservation of antigenic, unmodified peptides is key. Natural diversity in phagosome formation and fate may contribute to a balance between these needs.

5.3 Phagocytic Receptors

Receptors can participate in phagocytosis in several ways. Most basically, a phagocytic receptor binds to a particle (either directly or via opsonins) and initiates the intracellular signals that mobilize the cell to internalize the particle (Fig. 5.2). However, receptors that improve the efficiency or specificity of binding a particle to the cell surface (tethering receptors) and keep it from being swept away can be critical for efficient phagocytosis even if such receptors do not direct the intracellular signaling leading to internalization. Finally, as noted above, certain receptors can trigger

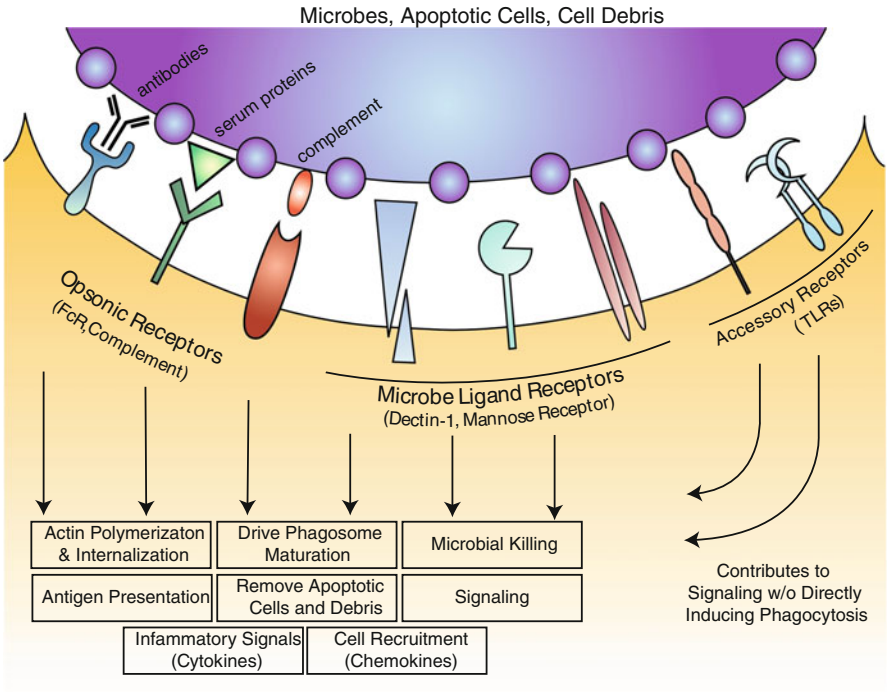

Fig. 5.2 Roles of receptors in phagocytosis. Diverse receptors participate alone or in combinations with each other to recognize particles. Receptors can recognize particles directly or they can recognize particles indirectly through opsonins bound to particle surfaces. Opsonic and microbial ligand receptors can trigger phagocytosis directly as well as contribute to diverse cellular responses to particles. In addition, "accessory receptors" may contribute to phagocytosis either by tethering particles and promoting their association with cells, or by enhancing signaling through other receptors

relatively non-specific uptake of particles or enhance the efficiency of signaling through the receptor driving internalization signals. We have identified a number of model receptor types above in our discussion of mechanisms of phagocytosis, and here we broaden our consideration of types of receptors involved in phagocytosis.

5.3.1 Fc Receptors

IgG-opsonized particles are recognized by several receptors that bind to the Fc region of IgG (FcγRs). Different phagocytes express different combinations of FcγRs, and all can contribute to initiating and regulating phagocytosis. These receptors can be classified as either "activating" or "inhibitory." As outlined above, the activating receptors contain ITAM motifs in their intracellular domains that recruit kinases and activate phosphorylation cascades driving phagocytosis

(as well as other immunomodulatory signals). Activating receptors include the high affinity FcγRI (CD64) and the low affinity FcγRIIA (CD32A) and FcγRIIIA (CD16). FcγRIIB (CD32B) is a low affinity inhibitory receptor that contains an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) that recruits the phosphatase SHIP, which blocks phosphoinositide signaling and antagonizes productive ITAM signaling. Thus, the relative expression of activating and inhibiting FcγRs determines the threshold for phagocytosis and inflammatory responses to IgG-opsonized particles.

While IgG may be the most prevalent type of antibody in serum, at mucosal surfaces IgA is the most common, and IgA-opsonized particles can be phagocytosed by cells expressing receptors for IgA. While a variety of receptors that bind IgA have been described, the one that is most relevant for discussions of phagocytosis is FcαRI (CD89) (Bakema and van Egmond 2011; Wines and Hogarth 2006). FcαRI is expressed widely by myeloid phagocytes, but is not present on certain cells such as mast cells, basophils, or intestinal macrophages. Although it is found in most mammals, the receptor is not found in mice due to a gene translocation. Like FcγRI and FcγRIIIA, FcαRI associates with the FcR common γ chain (FcRγ) which provides the ITAMs necessary for signaling. However, signaling via FcαRI is not modulated by the inhibitory FcγRIIB, and activity must be regulated differently. The receptor naturally has a low capacity to interact with IgA immune complexes, but "inside out" signaling upon, for example, cytokine stimulation of the cell dramatically increases its binding capacity and permits signaling via the receptor (Bakema and van Egmond 2011).

5.3.2 Lectin Receptors

In addition to the role of Dectin-1 in signaling phagocytosis of fungi discussed above, there are many other lectin receptors that can play roles in tethering various particles to cells if not directly inducing phagocytosis. Lectin receptors recognize carbohydrate motifs in microbial cell walls or in side chains of proteins and lipids that can be found in bacteria, fungi, virus, apoptotic cells, and extracellular matrix components. Perhaps the archetype of lectin phagocytic receptors is the mannose receptor. The mannose receptor is a type I transmembrane protein with multiple extracellular carbohydrate recognition domains and a short intracellular signaling tail. It is expressed widely on macrophages and dendritic cells, and expression of the mannose receptor in non-phagocytic Cos1 cells has been reported to confer the ability to phagocytose yeasts (Ezekowitz et al. 1990). Other similar type I transmembrane lectin receptors include Endo180 and Dec205, receptors that also have multiple extracellular lectin domains, but their abilities to directly trigger phagocytosis are not clear.

Dectin-1 is a type II transmembrane protein with a single extracellular lectin domain and a short N-terminal cytosolic domain. Dectin-1 is a member of a family of proteins with similar structures including Dectin-2, Mincle, DNGR-1, DC-SIGN,

and DC-SIGNR among others (Hardison and Brown 2012). However, other than Dectin-1, evidence for specific phagocytosis-triggering activity for these receptors is not available, and many may simply participate in binding to particles. In addition to being important for phagocytosis, lectin receptors such as Dec205 have been shown to enhance the delivery of diverse soluble antigens to intracellular compartments to enhance antigen loading on MHC molecules and subsequent T cell activation (Bonifaz et al. 2004).

5.3.3 Integrins

In addition to the β_2 integrins, which include complement receptors 3 and 4, a variety of other integrins have been demonstrated to be capable of directing phagocytosis of target particles. Particles can be opsonized with soluble extracellular matrix proteins such as fibronectin and vitronectin that facilitate phagocytosis through integrins such as $\alpha_V\beta_3$ and $\alpha_5\beta_1$. $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins interact with apoptotic cells through association with MFG-E8 secreted by macrophages and DC which interacts with phosphatidylserine but probably acts together with coreceptors like CD36 and CD14 to initiate phagocytosis. In addition to apoptotic cells, integrins play a maintenance role by engaging diverse extracellular matrix components and β-amyloid peptide complexes to internalize and clear debris (Bamberger et al. 2003).

5.3.4 Scavenger Receptors

Class A scavenger receptors are homotrimers characterized by extracellular collagenous domains, and they are able to bind directly to diverse microbial cell walls. Scavenger receptors tend to recognize polyanionic surfaces. That only certain polyanions are ligands for scavenger receptors suggests that there are both charge and structural requirements for binding, but these are not well understood (Peiser et al. 2002). Class A scavenger receptors include Scavenger Receptor A (SRA) and MARCO (macrophage receptor with collagenous structure). The signaling requirements for phagocytosis induced by scavenger receptors have not been worked out, and reports variously claim that the receptors are sufficient for phagocytosis (i.e., when ectopically expressed in non-phagocytic cells) or participate principally in binding to particles. In any case, it is clear that cells deficient in SRA or MARCO show marked defects in their abilities to phagocytose diverse targets including apoptotic cells and many types of bacteria (Bowdish and Gordon 2009; Greaves and Gordon 2009). It is likely these receptors play essential roles in binding and tethering microbes to phagocytic cells and, together with other phagocytic receptors, can have synergistic effects to enhance both uptake and downstream signaling (Mukhopadhyay et al. 2010). The class B scavenger receptor, CD36, also functions

as a non-opsonic phagocytic receptor and recognizes diacylated lipopeptides in microbial cell walls. A role for CD36 has been demonstrated in phagocytosis of bacteria and of *Plasmodium falciparum*-infected red blood cells, but as for the other scavenger receptors, the molecular mechanisms by which phagocytosis is triggered are poorly understood (Baranova et al. 2008; Patel et al. 2004; Stuart et al. 2005).

5.4 Physical Properties of Particles Affecting Phagocytosis

In addition to simple ligand–receptor interactions, larger physical features of a particle including size, shape, and rigidity can influence if and how a particle is phagocytosed. Actin-mediated phagocytosis is typically restricted to particles between 0.2 and 5 μm in size, and optimal internalization has been shown for opsonized model polystyrene beads of 2–3 μm in size (Champion et al. 2008). This presumably allows the particle to engage and cluster a threshold number of receptors to induce actin polymerization and trigger formation of membrane ruffles of a scale that is optimal for contact with particles in this size range (Doshi and Mitragotri 2010). Although most studies examining the effects of particle size make use of round polymer beads, microbes vary significantly in their shape and often have non-uniform surfaces. For example, many bacteria have elongated rod shapes, and budding yeast cell walls are pock-marked with bud and birth scars.

In order to specifically model the role of shape during phagocytosis, several groups have produced polymeric beads with various defined shapes. One such study indicates that orientation of particle contact can be even more important than overall size. When confronted with an oblong particle, the contact site, be it a side or a narrow end, is bound and actin polymerization is induced. However, extension of the actin-driven membrane ruffle around the particle is highly inefficient if the particle is bound sideways and presents a very "wide" face (Champion and Mitragotri 2006). The behavior is similar to "frustrated phagocytosis," a term coined to describe a macrophage trying to "eat" a ligand-coated flat surface or a particle substantially larger than the cell. Also, efficient internalization seems to require the angle of membrane contact compared to the axis of the particle to be less than 45°, perhaps to allow actin polymers to be able to bend around the particle.

The "stiffness" of a particle can also influence the way a phagocyte interacts with it. Beads with the same general chemical composition but with slightly different cross linker concentrations to modify the "stiffness" show differing rates of internalization, with stiffer beads being more readily eaten (Beningo and Wang 2002). Such stiffness is likely sensed through tension generated on the actinomyosin cytoskeleton. This tension, or mechanosensing, can also be important for converting receptor-independent binding by membrane lipids into signals for particle internalization. Interactions between lipids and uric acid crystals lead to strong binding that, through mechanisms requiring actin polymerization and Syk, can drive inflammatory signals (Ng et al. 2008).

5.5 Information Gathering During Phagocytosis

It would be the rare situation in which phagocytosis involves the engagement of a single type of ligand by a single receptor. Bacteria, yeast, apoptotic cells, and other biological particles are recognized simultaneously by multiple phagocytic and tethering receptors, and they all contribute to the efficiency of internalization and development of an appropriate inflammatory response. In addition, by exposing particles to reactive oxygen, low pH, and hydrolytic enzymes, the process of phagocytosis reveals additional ligands that may interact with phagosomal and cytosolic receptors that contribute to the inflammatory response (Fig. 5.3). Thus a phagocyte gathers many types of information about a particle upon contact and during internalization.

Redundancy in receptors and mechanisms for recognizing particulate targets is inherently valuable. First, redundancy in microbial recognition methods makes it difficult for pathogens to avoid being recognized. Many pathogens modify their surface molecules in ways that seem devised to try to evade immune phagocytes.

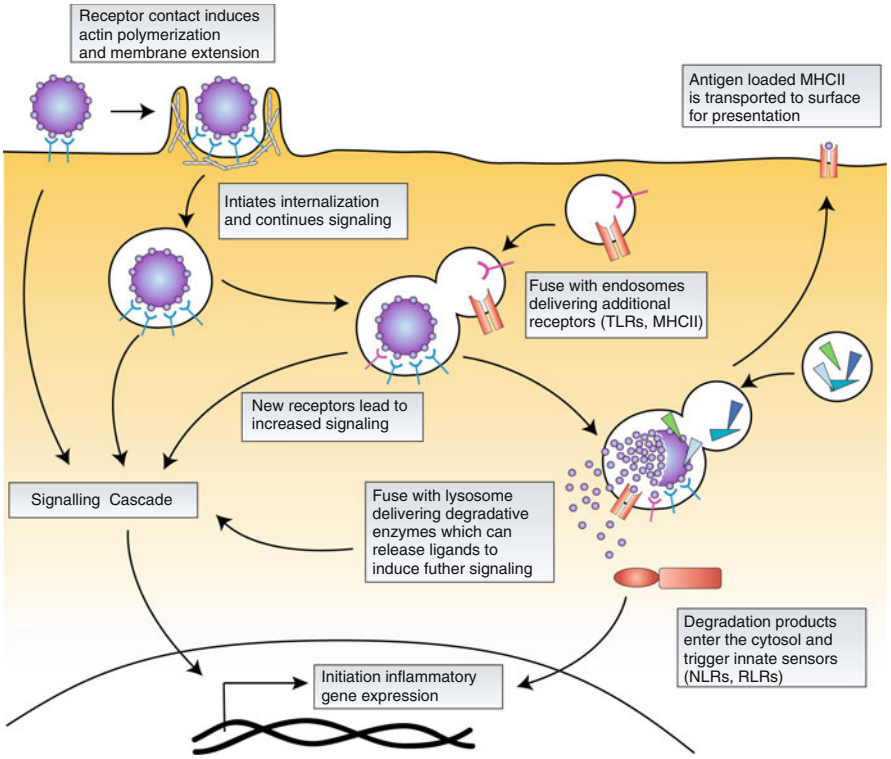

Fig. 5.3 Information gathering during phagocytosis. Information about a target particle can be acquired at many stages during the process of recognition, internalization, and degradation

For example, *Staphylococcus aureus* produces protein A to bind antibody Fc regions and thwart Fc receptor-mediated phagocytosis (Foster 2005). Similarly, *Cryptococcus neoformans* produces a thick polymer shell that masks cell wall ligands and suppresses phagocytosis (Garcia-Rodas and Zaragoza 2012). Second, redundancy is also good from a host genetics point of view. The more different receptors a cell has that can recognize a particular microbe, the less chance that a single genetic variation will allow a pathogen to escape detection.

Diversity in the types of mechanisms used to recognize a particle is also valuable. The use of different receptors to recognize a particle allows different information to be processed in order to evoke an appropriate cellular response. At the most basic level, we have already noted above that the mechanism of internalization triggered can be different depending on the receptor engaged. The differences in the types of responses, however, can run much deeper. Phagocytosis of certain particles (e.g., IgG-opsonized particles) is accompanied by activation of the NADPH oxidase and production of reactive oxygen species, while others (e.g., apoptotic cells) are not. Phagocytosis involving certain receptors such as CLEC9A (also known as DNGR-1) or DC-SIGN can promote cross-presentation on MHC class I (Sancho et al. 2009; van Kooyk et al. 2012). In contrast, activation of phagocytosis via Dectin-1 can increase the efficiency of MHC class II antigen presentation (Ma et al. 2012).

Diversity in the types of receptors engaged by a particle can also be an important part of decoding the relative level of danger posed. For example CD36 (Hoebe et al. 2005; Savill et al. 1992) and SRA (Suzuki et al. 1997; Platt and Gordon 1998) contribute to recognition of both apoptotic cells and pathogens. Dying cells are a normal part of embryogenesis, cessation of inflammatory processes, as well as tissue remodeling and repair, and their internalization is typically not inflammatory (Fadok et al. 1998; Voll et al. 1997). Thus, dying cells (not dangerous) need to be distinguished from pathogens (dangerous) by additional signals.

The process of phagocytosis itself can be an integral determinant in the nature and strength of the inflammatory response activated by a particle. For example, different TLR4 signaling mechanisms are activated depending on the cellular location of the receptor. Recognition of Gram-negative bacteria or LPS at the cell surface by TLR4 leads to engagement of the signaling adaptor molecule MyD88 and subsequent activation of NFκB and secretion of TNFα. However, once the phagosome forms and TLR4 is internalized, it engages the signaling adaptor molecule TRIF, leading to activation of IRF7 and IFNβ transcription (Kagan et al. 2008). Similarly, some receptors are regulated during internalization by becoming deactivated. Dectin-1 is activated by engagement of β-glucan-containing particles at the cell surface and triggers inflammatory signaling and phagocytosis. That the receptor is deactivated during internalization is demonstrated by the observation that cells produce elevated levels of cytokines (e.g., TNFα, IL-6, IL-12, IL-2) during "frustrated phagocytosis" in which cells try to ingest β-glucan particles too large to internalize, or in which particle internalization is blocked with cytochalasin D (Rosas et al. 2008).

The process of phagocytosis itself can also be an integral part of the inflammatory response by promoting the release of ligands that are then detected either by additional receptors in phagosomes or by receptors in the cytosol after transport across

the phagosome membrane. While surface TLRs are actively recruited to newly forming phagosomes, this is not dependent on detection of ligands (Ozinsky et al. 2000; Underhill et al. 1999). Instead, it appears that the receptors are poised to detect ligands should they become revealed during the process of phagocytosis. Indeed, activation of TLR2 during internalization of *S. aureus* is significantly abrogated if phagosome maturation, acidification, or the activities of hydrolytic enzymes are blocked (Ip et al. 2010; Wolf et al. 2011). In addition, intracellular TLRs (e.g., TLR9, TLR3) that are not typically expressed at the cell surface are nevertheless trafficked to maturing phagosomes where they can detect release of microbial nucleic acids as cell walls are broken down (Kasperkovitz et al. 2010; Schulz et al. 2005).

In the cytosol, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are poised to recognize microbial ligands. While these families of receptors recognize bacteria and viruses that gain access to the cytosol, there is also evidence that they can recognize the products of degradation generated in phagosomes (Charriere et al. 2010; Herskovits et al. 2007). Blockade of phagosome maturation, acidification, and degradation has been shown to suppress activation of the cytosolic NLRP3 inflammasome complex in response to *S. aureus* (Shimada et al. 2010). In most cases, the exact transporters used to deliver degradation products into the cytosol have not been directly investigated, but mature phagolysosomes are known to have transporters for sugars, amino acids, peptides and lipids as part of their normal function in metabolite recovery and recycling. Additional mechanisms that have been suggested include active transport by bacteria of products into the cytosol (Viala et al. 2004) and non-specific release due to leakage or phagosome disruption (Sander et al. 2011).

Signals generated during phagocytosis dictate the fate of the organelle, directing it for enhanced antimicrobial activity, enhanced antigen processing and presentation, or anti-inflammatory/silent handling of cargo. Regulation of these processes is especially critical given the essential role that macrophages and dendritic cells play in the removal of dying cells of both necrotic and apoptotic origins. Because dying cells arise during both normal tissue homoeostasis as well as during microbial infections, it is essential that phagocytes possess mechanisms for distinguishing normal cell turnover from cell death associated with infection and orchestrate the level of associated inflammation accordingly. One way cells distinguish danger from non-danger is through the phagocytic receptors that are engaged. For example, maturation of phagosomes containing unopsonized apoptotic cells internalized by CD36 is faster than that of phagosomes containing IgG-opsonized particles internalized by Fc receptors. While MHCII molecules are recruited to both phagosomes, the rapid phagosome maturation and associated antigen degradation lead to inefficient antigen loading (Blander and Medzhitov 2006). However, this block may be overcome by the addition of a TLR stimulus to the phagosome. In addition, apoptotic cell engagement by phagocytes reduces inflammatory responses even during an infection; LPS-stimulated macrophages fed apoptotic cells switch from proinflammatory TNFα production to anti-inflammatory IL-10 and TGFβ production (Fadok et al. 1998; Voll et al. 1997). Yet dying cells are not always anti-inflammatory; dendritic cells that have phagocytosed influenza-infected dying cells are able to cross-present antigen on MHCI to CD8 T cells leading to immune activation (Albert et al. 1998).

Similarly, Th17 T cell activation has been seen when dendritic cells internalize *Escherichia coli*-infected cells (Torchinsky et al. 2009), but this process must be tightly regulated to avoid autoinflammation from the presentation of self-antigens.

Mechanisms by which signaling on phagosomes directs the nature of the maturation of the compartment in order to orchestrate subsequent inflammation are still being elucidated. One mechanism that is emerging is the process of "LC3-associated phagocytosis." Light Chain 3 (LC3) is a microtubule-associated protein that is highly conserved across species. While well-known for its role in autophagy, recent findings have suggested that it may play a related but distinct role in the process of traditional phagocytosis. In 2007, Green and coworkers found that phagosomes in which TLR2 signaling was activated recruited GFP-tagged LC3 (Sanjuan et al. 2007). The investigators confirmed by electron microscopy that the organelle coated with LC3 was indeed a traditional phagosome enclosed in a single membrane, not the double-membrane compartment formed during autophagy. These investigators noted that LAMP1 recruitment was delayed and killing of microbes was impaired in cells lacking autophagy signaling components. More recently, Ma et al. demonstrated that Dectin-1 signaling is sufficient to direct LC3 recruitment to phagosomes and that this is directly driven by reactive oxygen production on phagosomes. Further, LC3 recruitment was demonstrated to be required for optimal MHCII antigen presentation, since this process was less efficient in cells lacking LC3 (Ma et al. 2012). These results suggest that not only does autophagy play a role in handling cytosolic bacteria, but autophagy-associated proteins are likely to play important roles in traditional phagocytosis as well.

5.6 Conclusion

In its most basic sense, phagocytosis is simply the process by which a cell eats another particle. However, in the context of mammalian phagocytes and host defense, the process of phagocytosis is an integral part of how the immune system gathers information about the nature of a threat. Diverse intracellular signals are generated during phagocytosis that direct the internalization of the particle, its degradation, activation of various anti-microbial killing processes, and production of appropriate inflammatory or anti-inflammatory mediators. Deeper understanding the mechanisms underlying the relationship between phagocytosis and inflammation should advance the development of treatments for inflammatory diseases, new approaches to vaccine delivery, and rational strategies for the treatment of infectious diseases.

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Chapter 6
The Antimicrobial Functions of Macrophages

Ronald S. Flannagan

6.1 Introduction

Macrophages are professional phagocytes and are thus uniquely endowed with the ability to ingest large particulates, including microorganisms. The process whereby macrophages engulf microbial prey is termed phagocytosis and it is central to their immunity-related functions. Through phagocytosis macrophages destroy microbial invaders and subsequently display at their cell surface prey-derived antigens to educe adaptive immunity. To further shape immune reactions macrophages stimulated with microbial products produce cytokines that promote additional leukocyte recruitment to sites of infection. To exert these immunomodulatory functions macrophages must prevail over would-be pathogens and this is achieved through the strategic deployment of a sophisticated antimicrobial armamentarium. While phagocytosis and the eradication of microbes is a habitual occurrence it is important to realize that "successful" pathogens have evolved strategies to combat the antimicrobial functions of macrophages.

6.2 Phagosome Maturation

Regardless of the phagocytic receptors that educe engulfment, in all instances phagocytosis culminates in the formation of a membrane-bound vacuole or phagosome. Initially the nascent phagosome is innocuous as its limiting membrane is a reflection of the plasmalemma from which it was derived and its fluid phase

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resembles the extracellular milieu. As such, internalization alone is insufficient to mediate microbial killing; however, after scission the nascent phagosome is rapidly remodeled acquiring distinct antimicrobial properties. This remodeling termed "phagosome maturation" is driven by a series of strictly choreographed membrane fission and fusion events between phagosomes and compartments of the endosomal/lysosomal network (Fig. 6.1). The molecular events that drive phagosome

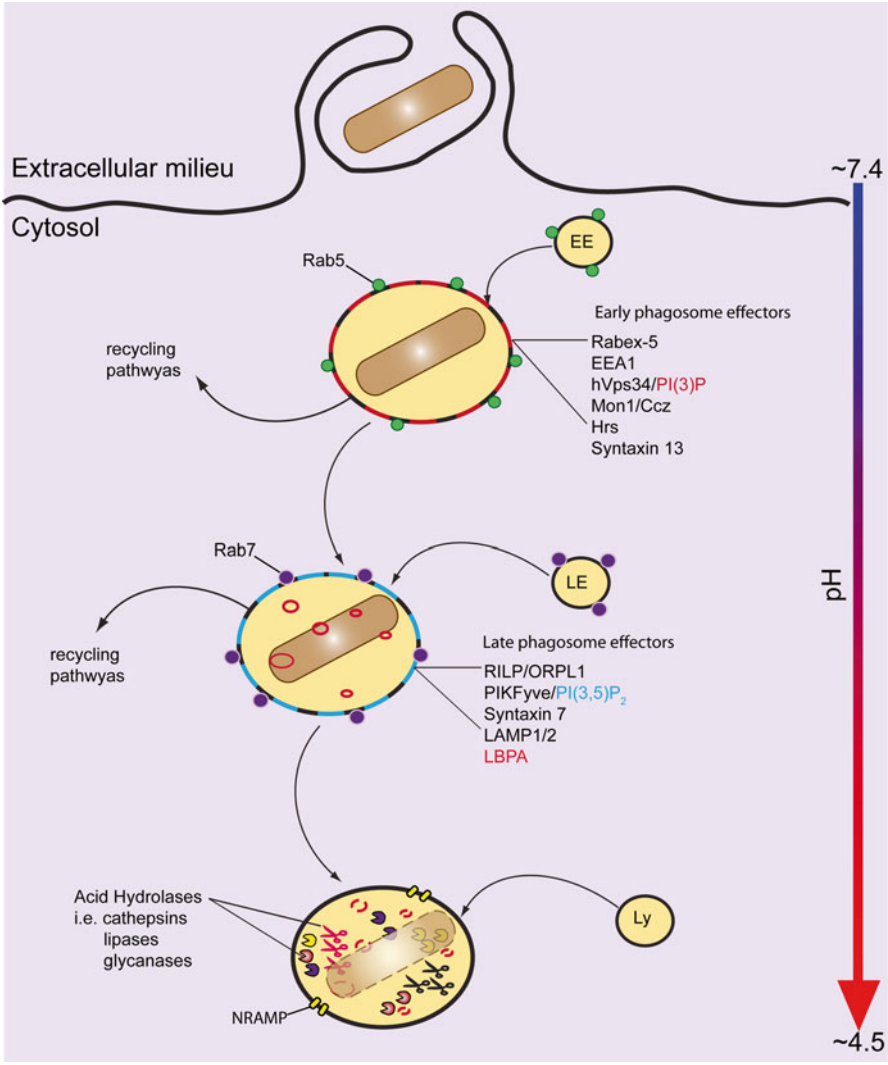

Fig. 6.1 Stages of phagosome maturation. Upon the engulfment of particulates, newly formed phagosomes undergo rapid remodeling to create degradative organelle with potent microbicidal properties. Through sequential interactions with early and late compartments of the endocytic

maturation are poorly defined but in recent years significant advances toward understanding how these dynamic organelles "mature" have been made. Some important observations have emerged from investigating phagosomes directly, however, others derive from the study of endosomes, a related but distinct archetype of membrane remodeling. Additionally, employment of model organisms such as Drosophila, yeast, and *Caenorhabditis elegans* has also greatly facilitated discovery. To date more than mammalian 20 phagocytic receptors have been described; however, analyses of Fcγ receptor driven phagocytosis have dominated the field. For this reason the proceeding discussion of phagosome maturation pertains primarily to FcγR-driven engulfment of phagocytic prey but it must be noted that important differences in phagosome remodeling are likely to occur depending on the identity of the receptors evoked.

6.3 The Early Phagosome

Transformation of the nascent phagosome occurs precipitously upon scission from the plasma membrane producing an early compartment that has undergone marked biochemical changes such as luminal acidification (~pH 6.5) (Desjardins et al. 1994). These changes in phagosome composition are a consequence of membrane traffic to and from the vacuole, a process that is coordinated by the family of molecular switches called the Rab GTPase proteins. Like other GTPases, Rabs cycle between active (GTP-bound) and inactive (GDP-bound) states, which is controlled by their cognate GDP-exchange factors (GEFs) and GTPase activating proteins (GAPS), respectively (reviewed in Stenmark (2009)). In their active state Rab proteins associate with effector proteins to influence distinct cellular processes that occur at the phagosome such as motor-driven vesicular traffic, membrane fission and fusion events, lipid metabolism, and even Rab activation.

On the early phagosome the Rab GTPase, Rab5, plays a pivotal role in regulating remodeling (Vieira et al. 2003). Without delay nascent phagosomes acquire Rab5, which is activated by its cognate GEFs, such as Rabex-5 (Horiuchi et al. 1997). On early endosomes Rabex-5 activates Rab5 to promote the recruitment Rabaptin-5 a Rab5 effector. As part of a feed-forward activation loop the Rab5/Rabaptin-5 complex promotes Rabex-5 GEF activity to foster further Rab5 activation (Lippé et al. 2001).

Fig. 6.1 (continued)Stage pathway phagosomes acquire distinct biochemical properties that are characteristic of early phagosomes, late phagosomes, and phagolysosomes. Through fusion events maturing phagosomes acquire the v-ATPase that pumps protons into the phagosome lumen to dramatically decrease its pH. Furthermore, as the phagosome matures it becomes enriched with hydrolytic enzymes and other antimicrobial proteins such as NRAMP, an H^+-dependent divalent cation metal transporter. *EE* early endosome, *LE* late endosome, *Ly* lysosome, *EEA1* early endosome antigen 1, *PI(3)P* phosphatidylinositol-3-phosphate, *Hrs* hepatocyte growth factor-regulated tyrosine kinase substrate, *LAMP* lysosome-associated membrane protein, *ORPL1* oxysterol-binding protein-related protein 1, *RILP* Rab7-interacting lysosomal protein, *LBPA* lysobisphosphatidic acid

Proteomic analysis of the early phagosome reveals that it is cohabited by Rab5, Rabex-5, and Rabaptin-5 where they ostensibly participate in a similar regulatory loop (Boulais et al. 2010). Regardless of how Rab5 is activated, it is clear that this GTPase plays a central role in regulating early phagosome morphogenesis by promoting early endosome fusion with the immature phagosome (Vieira et al. 2003). The fusogenic function of Rab5 is exemplified by the fact that heterologous expression of a mutated Rab5 allele that is GTP-locked induces the formation of giant early endosomes and phagosomes that fail to mature (Duclos et al. 2003). To regulate phagosome maturation Rab5 employs multiple effectors including the proteins APPL1 (adaptor protein containing pleckstrin-homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin–amphiphysin–rvs domain 1), EEA1 (Early Endosome Antigen 1), the p15-/Vps34 complex, and Mon1a/b.

APPL1 is an early endosomal adaptor molecule that is recruited to membranous compartments via Rab5 (Miaczynska et al. 2004). Recent analyses of phagosome maturation reveal that APPL1 also resides on the limiting membrane of the early phagosome where, in a Rab5-dependent manner, it recruits the inositol 5-phosphatases Inpp5b and OCRL (Bohdanowicz et al. 2012). Phosphoinositides are essential signaling lipids that are integral to both phagosome formation and maturation. The phosphoinositide phosphatidylinositol-4,5-*bis*phosphate (PI(4,5,)P2), which is constitutively synthesized on the inner leaflet of the plasmalemma is depleted from the nascent phagosome a process that is partly dependent on OCRL and Inpp5b (Bohdanowicz et al. 2012). Interestingly APPL1-dependent recruitment of these phosphatases and the ensuing depletion of 5′ phosphorylated phosphoinositides attenuate Akt-dependent signal transduction from the phagosome (Bohdanowicz et al. 2012). Akt (protein kinase B) is a serine threonine kinase that when activated initiates pro-survival signaling cascades that is strictly regulated (Manning and Cantley 2007). Indeed, Akt activation during the phagocytosis of pathogens must be appropriately coordinated if a proper immune response is to ensue. During pathogen engulfment insufficient Akt activation could lead to macrophage cell death before normal immune related functions have been achieved, while over-activation of Akt might curb appropriately timed macrophage death to provide a niche where intracellular pathogens could thrive. To facilitate evasion of these disastrous scenarios the Rab5 effector APPL1 dampens, through OCRL and Inpp5b, Akt activation to presumably combat intracellular pathogens such as *Salmonella typhimurium*, that usurp Akt pro-survival signaling for their own benefit (Knodler et al. 2005; Faherty and Maurelli 2008). Currently this APPL1 paradigm applies to FcγR-driven phagocytic events and its role on phagosomes derived through other phagocytic receptors remains unaddressed. Ostensibly differences do exist as evidenced by complement receptor derived phagosomes that undergo a secondary wave of $PI(4,5)P_2$ synthesis that indirectly may enhance vacuole maturation (Bohdanowicz et al. 2010).

The class III phosphatidyl insositol 3-kinase Vps34 is also recruited to the early phagosome in a Rab5-dependent manner where it synthesizes phosphatidylinositol-3-phosphate (PI(3)P) from phosphatidylinositol (Vieira et al. 2001). Perturbation of Vps34 function ablates phagosome maturation indicating Vps34 and its product PI(3)P are pivotal to phagosome morphogenesis (Araki et al. 1996). The catalytic activity

of Vps34 is enhanced by the Vps15-like serine/threonine kinase p150 that stably associates with Vps34 and possibly facilitates its recruitment to the early phagosome through Rab5 association (Vieira et al. 2001; Murray et al. 2002). While Rab5 activation is necessary for Vps34-dependent production of early phagosomal PI(3)P, Rab5 acquisition and activation is entirely Vps34 independent. As such Rab5, as a central regulator of early phagosome biogenesis, exploits Vps34 to effect local PI(3)P synthesis and direct the acquisition of additional maturation factors. This localized production of a signaling lipid highlights the sophisticated mechanisms in place at the phagosome to effect appropriate spatio-temporal coordination of maturation.

The marked accumulation of PI(3)P on the cytoplasmic face of the early phagosomal membrane facilitates the recruitment of PI(3)P binding proteins such as the p40 subunit of the NADPH oxidase, EEA1 (Early Endosomal Antigen 1), and Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (Fratti et al. 2001; Tian et al. 2008). EEA1 is one protein that accumulates prominently on the early phagosome where it binds directly active Rab5 and is thus a Rab5 effector (Simonsen et al. 1998). Tethering of EEA1 to the early phagosome is also enhanced by its capacity to engage PI(3)P where it promotes docking and fusion of early endosomes (Simonsen et al. 1998; Christoforidis et al. 1999). This fusogenic function of EEA1 stems, in part, from its ability to interact with SNARE (soluble *N-ethylmaleimide-sensitive* factor attachment protein receptor) proteins such as syntaxin 13 (McBride et al. 1999). SNAREs are transmembrane proteins that drive membrane fusion throughout the endocytic network and do the same for maturing phagosomes. They function by forming hairpin-like complexes comprised of v-SNAREs that are present on donor membranes (e.g., early endosomes) and t-SNAREs that reside in acceptor membranes (e.g., phagosomes). Through v-SNARE/t-SNARE complex formation, donor and acceptor membranes come into direct apposition, which helps overcome the free-energy barrier that impedes spontaneous membrane fusion. Not surprisingly SNARE function is also subject to regulation and is controlled by the *N*-ethylmaleimide-sensitive factor or NSF. Indeed, perturbation of NSF or of SNARE function (i.e., syntaxin 13) arrests phagosome maturation emphasizing the notion that coordinated membrane fusion drives phagosome morphogenesis (Coppolino et al. 2001). Despite repeated rounds of membrane fusion and appreciable changes in the biochemical properties of nascent phagosomes their size remains relatively uniform. Implicit in this observation is that there must be a concomitant egress of membrane from the maturing phagosome. Indeed, an entire endosomal network of tubules and vesicles operates simultaneously to retrieve proteins such as transferrin receptor from the maturing phagosome. This recycling pathway is in part co-coordinated by the Rab GTPases Rab4 and Rab11 among other proteins (Damiani et al. 2004; Cox et al. 2000). While these protein retrieval mechanisms per se are not directly associated with the antimicrobial function of phagosomes the importance of the recycling pathway is not to be discarded as perturbation of recycling adversely affects phagosome maturation (Cox et al. 2000).

While egress of membrane from the phagosome contributes to the maintenance of phagosome size it is not the sole mechanism at play. Phagosomes also direct membrane-associated cargo destined for degradation to intraluminal vesicles (ILVs)

that are formed through invagination of the limiting phagosomal membrane. ILV formation on the phagosome ostensibly follows a process that is akin to multivesicular body (MVB) formation in the endosomal network. On endosomes MVB formation is catalyzed by the ESCRT (endosomal sorting complex for transport) machinery (reviewed in McCullough et al. 2013). Proteins destined for degradation through ILV formation are marked by mono or poly-ubiquitylation and these post-translational modifications facilitate cargo recognition and sorting (Katzmann et al. 2001). For instance the FcγRIIA receptor is ubiquitylated post-phagocytosis and this modification directs phagosome-associated receptor into ILVs for its eventual degradation (Lee et al. 2005). Sorting of ubiquitylated cargo requires the protein Hrs, which localizes to phagosomes at an early stage that precedes late phagosome and phagolysosome formation (Vieira et al. 2004; Pons et al. 2008). Hrs, a FYVE-domain containing protein is recruited to early PI(3)P enriched phagosomes where it presumably recognizes ubiquitylated substrates and participates in the early steps of ILV genesis (Vieira et al. 2004; Pons et al. 2008; Bache et al. 2003). Apparently Hrs-dependent processes at the phagosome are critical to maturation as experimental depletion of cellular Hrs in macrophages causes phagosomes to retain early markers of maturation for inordinate duration, causes inadequate phagosome acidification, and prevents the acquisition of late stage markers of phagosome maturation such as lyso-bis phosphatidic acid (LBPA; see below) (Vieira et al. 2004).

6.4 The Late Phagosome

Sustained remodeling of the early phagosome produces a late phagosome that is more acidic than its early predecessor (~pH 5.5–6.0). A hallmark change signifying the transition of early to late stage phagosome maturation is the acquisition of the Rab GTPase Rab7 with the concomitant loss of Rab5 (Vieira et al. 2003). The acquisition of active Rab7 is needed for phagolysosome formation as Rab7, through its effectors, directs phagosomes centripetally along microtubules to promote phagosome/lysosome fusion (Jordens et al. 2001; Harrison et al. 2003). The conversion of Rab5 to Rab7 has been heavily scrutinized and some progress towards elucidating the molecular machinery that mediates Rab conversion has been realized. Interestingly, Rab5 is not only a central regulator of early phagosome remodeling it also, through indirect means, stimulates the acquisition of Rab7 (Vieira et al. 2003). While much of the work to define this process stems from the characterization of endosomal and phagosomal fusion in yeast and *C. elegans*, mammalian phagosomal systems presumably operate analogously. In yeast Rab5 to Rab7 conversion requires the activity of the two Vps-C protein complexes, CORVET (class C core vacuole/endosomal tethering) and HOPS (homotypic fusion and vacuolar protein sorting) (Peplowska et al. 2007; Plemel et al. 2011). Through Rab5 CORVET is recruited to endosomal membranes where it is morphed into HOPS by replacement of the CORVET proteins Vps3 and Vps8 by the HOPS proteins Vps39 and Vps41, respectively (Plemel et al. 2011). Subsequent activation of Rab7 is reported to occur

through Vps39 of the HOPS complex, a purported Rab7 GEF (Wurmser et al. 2000). More recently, the Rab5 effector Mon1 and its binding partner CCZ1 were also found to directly engage Rab7 and influence its activation (Kinchen and Ravichandran 2010; Nordmann et al. 2010). Since Mon1 is also reported to bind the HOPS complex, Mon1 might employ multiple players to effect Rab7 activation including CCZ1 and/or HOPS (Kinchen and Ravichandran 2010; Poteryaev et al. 2010). Interestingly, Mon1 also displaces the Rab5 GEF Rabex-5 from mature early endosomes to presumably impede subsequent Rab5 activation, and ostensibly this would occur on phagosome too (Poteryaev et al. 2010). Precisely how Mon1/CCZ and possibly HOPS mediate Rab conversion remain incompletely defined but it is probable that each of these components coordinate the process of Rab7 activation.

Regardless of how Rab7 is activated it is a central regulator of late phagosome maturation and while the acquisition of active Rab7 is necessary for phagolysosomal fusion it is not sufficient. Obstruction of the class III PI3-kinase Vps34 also ablates phagolysosome fusion despite the fact that kinase inhibition does not preclude phagosomal recruitment of GTP-bound Rab7 (Vieira et al. 2003). Implicit in this observation is that, as of yet, unidentified phosphoinositide-dependent regulators of phagosome maturation must act in concert with Rab7 to yield phagolysosomes. In this regard, PIKfyve is a 5′ phosphoinositol kinase that catalyzes the formation of PI(3,5)P2 from PI(3)P in the endocytic pathway and it is also recruited to phagosomes (Kerr et al. 2010; Hazeki et al. 2012). Interestingly, in impairment of PIKfyve function interferes with membrane fusion between lysosomes and macropinosomes and presumably phagosomes explaining why PIKfyve perturbation diminishes the microbicidal capabilities of *S. typhimurium* infected macrophages (Kerr et al. 2010).

Despite the importance of Rab7 its effectors remain largely uncharacterized. Two proteins that associate in a Rab7-dependent manner with late endosomes, late phagosomes, and lysosomes are RILP (Rab-interacting lysosomal protein) and its partner ORPL1 (oxysterol-binding protein-related protein 1) (Jordens et al. 2001; Johansson et al. 2007). Together RILP and ORPL1 coordinate microtubule driven traffic of Rab7-bearing compartments by coupling them to the molecular motor dynein/dynactin. This consequential motor-driven centripetal displacement and tubulation of Rab7-positive phagosomes facilitates lysosome fusion, which may in part be driven through a Rab7/HOPS-dependent process (Van der Kant et al. 2013). As mentioned, Rab7 reportedly associates directly with the HOPS complex and HOPS is also required for lysosomal fusion with endosomes. Recent evidence from Drosophila studies demonstrates that depletion of the HOPS subunit Vps16 compromises the transition of late phagosomes to phagolysosomes resulting in immune defects in response to *Escherichia coli* infection in the fly (Akbar et al. 2011). In addition to Rab7 other GTPases also seemingly play an important role in phagolysosome formation. Rab20 another Rab GTPase has recently been shown to associate with the early phagosome but maintains its presence on phagosomal membranes through late phagosome stages (Egami and Araki 2012). How Rab20 influences phagosome maturation and the identity of its effectors remain enigmatic and await clarification.

While the role of Rab7 in late phagosome and phagolysosome formation is unquestionable other necessary factors also exist. The Lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2) are heavily glycosylated transmembrane proteins that are enriched in late endosomes, late phagosomes, and lysosomes. Previously these proteins were thought to play structural role involved in the maintenance of lysosomal integrity however, this tacit assumption has been proven invalid. It is now clear that without LAMP proteins phagosomes lose their microbicidal capacity (Huynh et al. 2007; Binker et al. 2007). Indeed LAMP-deficient cells harboring *Neisseria gonorrhea* fail to eradicate phagocytosed bacteria in contrast to cells with wild type LAMP expression which effectively kill phagocytosed bacteria (Binker et al. 2007).

The late stage phagosome is also a dynamic organelle like its earlier predecessor and the egress and invagination of membrane via recycling and ILV formation is sustained. Within the lumen of late phagosomes are ILVs, which are characterized by the presence of the tetraspanin protein CD63 and the biochemically unique glycerophospholipid LBPA. LBPA is particularly enriched in ILVs and ostensibly contributes to their formation. Indeed in vitro liposome-based studies demonstrate that the presence of LBPA in an acidic environment can promote inward membrane budding akin to ILV formation (Matsuo et al. 2004). The importance of LBPA to phagosome maturation is presently unclear but it may influence cholesterol trafficking, which when impaired can have drastic affects on phagolysosome formation (Chevallier et al. 2008; Huynh et al. 2008). Along these lines, the treatment of macrophages with the drug U18666A an agent that perturbs cholesterol efflux-impedes phagolysosome formation despite the acquisition of Rab7 remains intact (Huynh et al. 2008).

Through the concerted effort of theses aforementioned effectors of late phagosome maturation maturing phagosome eventually fuse with lysosomal compartments to create the ultimate microbicidal organelle, the phagolysosome.

6.5 The Phagolysosome

The mature phagosome or phagolysosome is an incredibly noxious organelle that is acidic (~pH 4.5), oxidative, and is degradative towards proteins, carbohydrates, and lipids. Endowment of the phagosome with these biochemical properties is achieved through membrane fusion with preformed lysosomes and represents a terminal step in the maturation pathway. Fusion between phagosomal membranes and lysosomes requires, like other membrane-bound compartments, the activity of SNARE proteins such as syntaxin 7, SNAP-23, and VAMP7 (Sakurai et al. 2012; Collins et al. 2002; Ward et al. 2000). Notably syntaxin 7 localizes to late phagosomes and its impairment blocks phagolysosome formation (Collins et al. 2002). Also required for the delivery of lysosomes to phagosomes is the activity of the lysosome-associated GTPase arf-like protein 8b (Arl8b) (Bagshaw et al. 2006). Arl8b on lysosomal membranes interacts with its effector SKIP to engage the plus-end directed microtubule motor kinesin promoting lysosomal trafficking away from the

microtubule-organizing center (Rosa-Ferreira and Munro 2011). It is tempting to speculate that this Arl8-driven lysosomal traffic brings phagosomes and lysosomes into apposition to facilitate fusion. In addition, Arl8b engages the aforementioned HOPS complex to ostensibly effect phago/lysosomal fusion (Garg et al. 2011). The importance of Arl8b to phagolysosome function is highlighted in experiments where macrophages deficient for the GTPase are unable to combat bacterial infection (Garg et al. 2011). These experiments also highlight the importance of phagosome maturation and the deployment of an antimicrobial arsenal to the phagosome in order to eradicate infection.

6.6 LC3-Assisted Phagosome Formation

Macroautophagy (herein after termed autophagy), the process whereby cells engulf and degrade cytosolic constituents through de novo synthesis of an intracellular vacuole, is an evolutionarily conserved process intended to promote cell survival under conditions of nutrient deprivation. More recently however, it has been appreciated that autophagy or at least some of the autophagic machinery intersects the "conventional" phagosome and contributes to cellular immunity (Fig. 6.2).

In the first scenario the autophagic machinery operates as a fail safe and is called into action after something drastic has occurred to the phagosome because of its noxious cargo (i.e., pathogenic bacteria). For instance when bacteria such as *S. typhimurium* or *Listeria monocytogenes* are engulfed by macrophages into sealed phagosomes the bacteria can promote membrane disintegration gaining access to the cytosol (Zheng et al. 2009; Cemma et al. 2011; Birmingham et al. 2006). To combat these bacterial escape artists infected cells can evoke the autophagic machinery to synthesize isolation membranes (also known as phagohores) to quarantine the fleeting bacteria in a double-membraned autophagosome. Isolation membrane formation, indicated by the presence of the lipidated autophagy protein LC3, is instigated upon host cell perception of the bacteria by cytosolic danger receptors such as galectin-8 and presumably others (Thurston et al. 2012). Through galectin-8 signals, cytosolic exposed bacteria are eventually ubiquitylated facilitating the association of adaptor molecules like p62/SQSTM1 and NDP52 that promote the genesis of the ever-important isolation membrane (Zheng et al. 2009; Cemma et al. 2011).

Another scenario where autophagy intersects phagocytosis involves the recruitment of LC3 to maturing phagosomes but this occurs in the absence of overt signs of phagosomal damage. In this instance it seems that the catalyst for LC3-association with phagosomes is NADPH oxidase activation and the production of reactive oxygen species (Huang et al. 2009). Interestingly, LC3 in this instance is associated with a single lipid bilayer, the limiting phagosomal membrane, and not a conventional double membrane that is characteristic of autophagosomes. At present it is unclear if the association of LC3 to the oxidized phagosome is a consequence of LC3-positive membrane delivery or is the result of covalent ligation of LC3 with

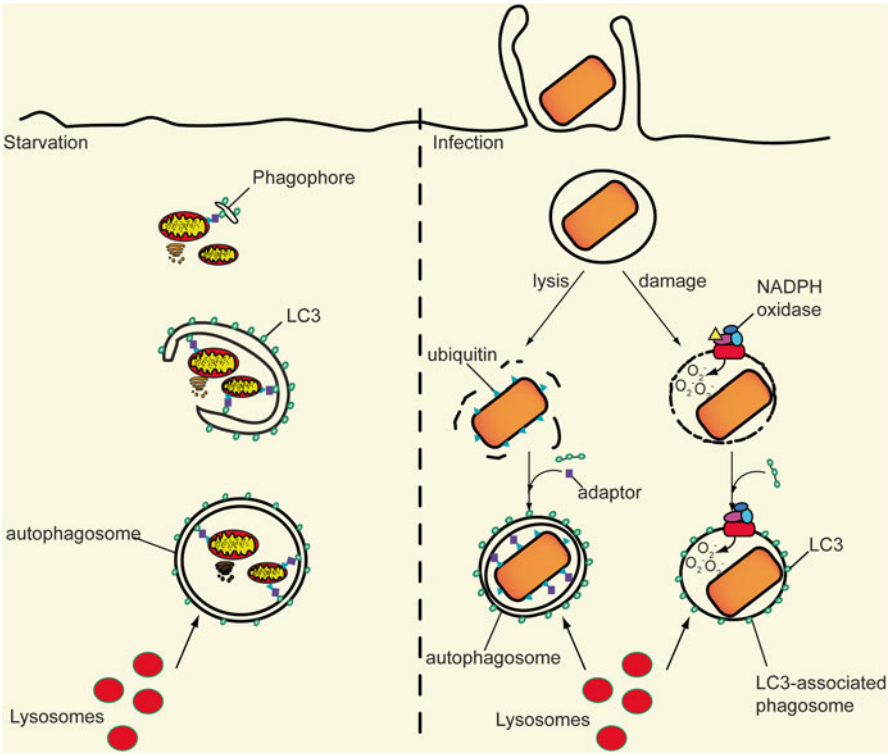

Fig. 6.2 LC3-associated phagocytosis. Macroautophagy and phagocytosis are similar in that both processes culminate with the formation of membrane-bound vacuoles. The tacit assumption that conventional phagocytosis and autophagy are mechanistically distinct is no longer tenable as autophagy related proteins participate in both processes. On the *left*, a schematic representation of a macrophage undergoing a starvation response and inducing macroautophagy is depicted. Cytosolic material (i.e., organelles, etc.) is ubiquitylated and recognized by adaptor proteins such as p62/SQTSM1 or NDP52. These adaptors in turn bind LC3 lipidated with phosphoethanolamine to locally recruit LC3-decorated isolation membranes. Upon fusion of the isolation membranes a vacuole with a double membrane is created giving rise to the autophagosome. On the *right*, a macrophage having phagocytosed a pathogenic bacterium is depicted. In the first scenario, the bacterium through the production of effector proteins causes phagosome lysis. The cytosolic exposed bacterium is subsequently perceived by the cell, which leads to its ubiquitylation and adaptor protein recognition. In turn LC3-positive isolation membranes are recruited resulting in the formation of a double membrane autophagosome that encloses the bacterium prior to lysosome fusion. Alternatively, LC3-associated phagocytosis is depicted (far *right*). In this scenario, during bacterial engulfment the production of a robust oxidative burst damages the phagosomal membrane without triggering overt lysis. This damage leads to the recruitment of LC3-positive membranes to ostensibly promote phagosome repair and ultimately lysosomal fusion. Importantly, during this process the characteristic double autophagosome membrane is absent

phosphatidylethanolamine in the cytosolic leaflet of the limiting phagosomal membrane. Regardless of how it is acquired, presumably LC3-association promotes phagosome repair and/or lysosomal fusion allowing for the formation of a microbicidal phagolysosome.

6.7 Antimicrobial Properties of the Phagolysosome

6.7.1 Phagosome Acidification

Immediately after scission from the plasmalemma the pH of the phagosome declines until it reaches a pH of ~4.5. Acidification is the consequence of the extrusion of protons into the phagosome lumen by the vacuolar ATPase (v-ATPase); a massive (~10^3 kDa) multi-subunit protein that pumps protons across the limiting phagosomal membrane at the expense of ATP (Jefferies et al. 2008; Lukacs et al. 1991). With the delivery of endosomal membranes to maturing phagosomes continuous acquisition of the v-ATPase occurs resulting in a massive influx of protons (Sun-Wada et al. 2009). Concomitantly the passive dissipation of protons, the so-called proton leak, across the limiting phagosomal membrane is diminished enhancing H^+ accumulation (Lukacs et al. 1991). As v-ATPase activity is strongly electrogenic additional ion transport processes must operate simultaneously to prevent the genesis of a prohibitive electrical potential across the phagosomal membrane. The inward flux of Cl^- counterions and the efflux of cations (i.e., Na+ or K+) serve this purpose and allow for sustained v-ATPase activity (Steinberg et al. 2010; DiCiccio and Steinberg 2011).

Proton pumping and phagosome acidification is essential to phagosome physiology. The low pH is inherently antimicrobial and can curtail microbial growth while simultaneously enhancing the degradative capacity of the phagosome (Ip et al. 2010). H^+ accumulation and phagosome acidification is also not merely a consequence of maturation but may also be a prerequisite for maturation to proceed. Indeed, in vitro experiments reveal that perturbation of proton pumping by pharmacological means or by alkalization using a cell permeant weak base impedes phagosome maturation without affecting engulfment (Gordon et al. 1980; Lukacs et al. 1990).

As a central parameter in phagosome physiology it is not surprising that acidification influences other antimicrobial processes in the phagosome. For instance, luminal protons indirectly impart phagosomal nutritional immunity by activating the NRAMP cation transport, an antimicrobial concept discussed further below.

6.8 Reactive Oxygen and Reactive Nitrogen Species

The importance of ROS as a host microbicidal defense is undisputable and made evident by individuals afflicted with the rare recessive genetic condition termed Chronic granulomatous disease (CGD). CGD patients experience serious recurrent fungal and bacterial infections due to impaired phagocyte NADPH oxidase or NOX2 activation (Bylund et al. 2005). NOX2 is comprised of multiple proteins that are integral to membranes (gp91^{phox} and p22^{phox}, together called cytochrome *b558*) and soluble proteins that exist in a cytosolic complex (p67^{phox}, p47^{phox}, p40^{phox}) (Lam et al. 2010). Upon the perception of activating signals cytochrome *b558*

interacts with $p67^{phox}$/$p47^{phox}$/$p40^{phox}$ at the forming phagosome, where association of GTP-bound Rac with $p67^{phox}$ enables the oxidase activation (DeLeo et al. 1999; Heyworth et al. 1994). Through the oxidation of NADPH the oxidase transfers electrons to molecular oxygen in the phagosome lumen producing the noxious super oxide anion O^-. O^- can subsequently be dismutated into hydrogen peroxide (H_2O_2), another toxic ROS, which can also be converted via Fenton chemistry to reactive hydroxyl radicals. In combination, NOX2 derived ROS represent a potent microbicidal facet of the macrophage antimicrobial response as these compounds lethally oxidize microbial carbohydrates, lipids, and nucleic acids.

On sealed phagosomes the NOX2 subunit $p40^{phox}$ is required for oxidase activation and its spatio-temporal distribution in the cell is influenced by Vps34 and its product PI(3)P (Tian et al. 2008). Indeed, $p40^{phox}$ is endowed with a PX lipid binding domain that recognizes phagosomal PI(3)P and thus modulating local PI(3)P production at the phagosome can have profound effects of ROS production (Tian et al. 2008). Immunomodulatory proteins like the signaling lymphocyte-activation molecule family (SLAMF) expressed by most hematopoietic cells including macrophages regulate phagosomal NOX2 activation in response to pro-inflammatory stimuli. SLAMF1, a member of the SLAM family, acts as a sensor of Gram-negative bacteria outer membrane proteins and modulates on the phagosome NOX2 activation through Vps34 activation and PI(3)P production (Berger et al. 2010).

Not all phagocytes are created equally with respect to their NOX2-dependent oxidative burst and while neutrophils mount the most robust NOX2 response, macrophages are not far behind. Interestingly important differences in NOX2 expression and activation can exist and are influenced by the macrophages alternative activation state. Along these lines, macrophages that are M2 polarized have a diminished capacity to produce ROS through NADPH oxidase activation owing to decreased expression of $gp90^{phox}$ (Balce et al. 2011). Furthermore, the phagolysosomes of M2 polarized macrophages also display enhanced proteolytic capacity, a phenotype attributed to increased cysteine cathepsin activation due to changes in phagosomal redox chemistry, which is in turn regulated by NOX2 activation (Balce et al. 2011; Rybicka et al. 2010). In this sense and akin to phagosome acidification, phagosomal ROS and NOX2 are integral components that modulate phagosome physiology.

Like ROS macrophages also produce at the phagosome reactive nitrogen intermediates (RNI) that also contribute to pathogen eradication by triggering structural damage to constituents of the microbial cell. Unlike ROS however, the production of RNI is delayed as expression of the enzyme required for their synthesis is repressed until inflammatory stimuli (i.e., lipopolysaccharide or interferon γ) are detected (Weinberg et al. 1995). The enzyme responsible for RNI synthesis is termed nitrous oxide synthase 2 or better known as inducible nitric oxide synthase (iNOS). Chemically iNOS extracts an electron from NADPH and oxidizes L-arginine to yield citrulline and the NO^- radical (Bogdan et al. 2000). This reaction occurs at the cytosolic face of the phagosomal membrane with the subsequent diffusion of NO^- into the lumen where it can react with phagocytic prey. While NO^- is highly toxic,

it, like ROS, is also converted into other microbicidal reactive nitrogen species such as peroxynitrite ($ONOO^-$). Together ROS and RNI comprise a potent antimicrobial armament that effectively kills intraphagosomal pathogens.

6.9 Effectors of Nutritional Immunity

Certain nutrients are essential for microbial growth and survival and limiting access to such nutrients is an effective antimicrobial strategy that is at play in the phagosome or in the cytosol of infected macrophages. For instance the acquisition of iron is essential for bacterial growth; however, due to the ejection of Fe^{2+} and other divalent cations from the phagosome lumen by the metal ion transport protein NRAMP, the phagosome lumen is depleted of these nutrients (Nairz et al. 2009). Interestingly, NRAMP function is tightly coupled to phagosome acidification as luminal H^+ provide the energy to NRAMP to mediate metal ion extrusion form the interior of the phagosome (Jabado et al. 2000, 2002). Controlling cellular iron is not a trivial matter and an additional axis to regulate the Fe content of macrophages operates during infection. Ferroportin 1 is a Fe^{2+} exporter that transports iron out of macrophages when the metal is plentiful (Ganz 2012). Interestingly recent data suggests ferroportin 1 expression is up-regulated by iNOS and NO^- production (Nairz et al. 2013). The importance of this is apparent when ferroportin 1 driven egress of iron from macrophages is suppressed as the ability of macrophages to control intracellular survival of bacterial pathogens is dampened (Nairz et al. 2007, 2013).

In addition to essential metals intracellular pathogens frequently usurp host cell biosynthetic processes for their own benefit and steal previously synthesized macromolecules for their own metabolism. To thwart being parasitized host cells will catabolize cellular stores of amino acids and nucleotides. SAMHD1 a protein expressed by macrophages and other myeloid cells is a nuclear triphosphohydrolase that in response to viral infection drastically depletes the intracellular pool of deoxynucleotide triphosphates (dNTPs) to limit viral genome replication (Pauls et al. 2013; Lahouassa et al. 2012). Similarly the interferon induced indoleamine-2,3-oxygenase (IDO) is an enzyme that degrades intracellular L-tryptophan to potently restrict the growth of bacteria (i.e., Francisella, Chlamydia, and Rickettsia), viruses (i.e., hepatitis B virus), and protozoa (i.e., Toxoplasma) that attempt to parasitize (MacMicking 2012).

6.10 Lysosomal Matrix Proteins

Fusion of phagosomes and lysosomes is an essential aspect of the macrophage innate defense and as a testament to this successful intracellular pathogens go to great lengths to perturb this process (reviewed in Sarantis and Grinstein 2012).

The toxicity of the macrophage lysosome can in part be attributed to its immense degradative capacity that can catabolize complex structures like dead or dying bacteria (Ip et al. 2010). Lysosomal degradation is the consequence of expressing greater than 60 acid hydrolases that function as peptidases/proteases, glucosidases, lipases, and amylases among others. Proteases belonging to the cathepsin family are perhaps the most widely studied lysosomal matrix proteins (Schröder et al. 2010). This family of enzymes is comprised of aspartate, serine, or cysteine proteases and they tend operate optimally under the conditions found in the lysosome (Turk et al. 2012). Additionally, their activity at least for cysteine cathepsin is also influenced by the lysosomal redox state controlled in part by NOX2 activation (Balce et al. 2011).

6.11 Other Macrophage Immunity-Related Proteins

In addition to phagocytosis and phagosome maturation, macrophages employ additional factors to control infection. Macrophages secrete soluble factors like cytokines that directly alter the antimicrobial properties of other immune cells and macrophages themselves. Furthermore, macrophages produce other proteins that directly attack structural components of microorganisms to cause microbial cell death. Interleukin 1β (IL-1β) and interferon-γ (IFN-γ) are potent pro-inflammatory cytokines that profoundly affect macrophage antimicrobial function. Indeed, IFN-γ stimulation induces the expression of several genes such as the IFN-γ-inducible 65-kD GTPases that are required for cellular immunity to bacteria and parasites (Kim et al. 2011; Yamamoto et al. 2012). IL-1β, produced in response inflammasome activation, also enhances the antimicrobial response of macrophages to promote the elimination of intracellular pathogens such as *Mycobacterium tuberculosis* (Jayaraman et al. 2013). In addition to cytokine production inflammasome activation can also effect pyroptosis, a pro-inflammatory process of cell death displayed by macrophages in response to intracellular infection (Aachoui et al. 2013; Lamkanfi and Dixit 2012). Interestingly by triggering their own cell death in response to microbial infection macrophages make the intracellular bacteria accessible to other phagocytes that may be better suited to combat the infection (Miao et al. 2010).

To exert direct attack on microorganisms, macrophages produce a variety of proteins that compromise the structural integrity of microbes. Lysozyme, a *N*-acetylmuramide glycanhydrolase that cleaves the β(1,4) glycosidic linkages in the peptidoglycan of Gram-positive and Gram-negative bacteria, is expressed by macrophages and other phagocytes (Ganz 2004). As a testament to the importance of lysozyme as an antimicrobial effector, bacteria like *Staphylococcus aureus* and *L. monocytogenes* have evolved peptidoglycan modifying enzymes that help protect these bacteria from lysozyme-dependent degradation (Rae et al. 2011; Shimada et al. 2010). Macrophages also produce antimicrobial proteins belonging to the cathelicidin family of antimicrobial peptides (Gombart et al. 2005; Pinheiro da Silva et al. 2009). Cathelicidins, like defensins which macrophages lack, compromise the integrity of

bacterial membranes to cause microbial cell death. Furthermore, cathelicidins are multi-talented proteins as they can also influence cellular processes such as autophagy in addition to possessing antimicrobial properties (Gombart et al. 2005).

6.12 Conclusions

The engulfment of microbial prey and their eradication through phagosome maturation is an essential facet of the macrophage antimicrobial response. This portal allows the macrophage to not only effect innate immune reactions but also enables bridging of these innate responses to adaptive immunity through antigen presentation. When microbial killing through phagocytosis fails other mechanisms of macrophage immunity such as pyroptosis come into effect to limit infection. While much has been learned about how macrophages exert their antimicrobial functions and how microbes evade these strategies, many more important findings await discovery.

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Chapter 7
Vascular Modulatory Functions of Macrophages

Ioanna Keklikoglou and Michele De Palma

7.1 Introduction

Angiogenesis is the process whereby new blood vessels arise from the pre-existing vasculature via sprouting or splitting, and are subsequently fused with other blood vessels, a phenomenon known as vascular anastomosis. Angiogenesis occurs during development and tissue/organ healing or regeneration, as well as in several pathological conditions such as cancer (Potente et al. 2011; Weis and Cheresh 2011).

Blood vessels supply tissues with nutrients and oxygen, and regulate the removal of metabolic waste products via the blood circulation. In order to gather blood supply, growing (avascular) tissues induce angiogenesis by activating a genetic program that is largely dependent on the vascular endothelial growth factor (VEGF)-A, whose expression is induced by low oxygen tension (hypoxia) through the stabilization of hypoxia-inducible factors (HIFs). Hypoxia also stimulates the recruitment, activation and/or retention of vascular-modulatory cells, such as macrophages and neutrophils. These cells release proangiogenic mediators and other growth factors that enhance or otherwise support the local angiogenic response triggered by VEGFA (Potente et al. 2011; Weis and Cheresh 2011).

Macrophages play important functions as innate immune cells, as they constitute a first line of defense against invading pathogens. Indeed, they phagocytose microbes and can present antigens to T-cells to promote adaptive immune responses. Macrophages also express a broad array of chemokines, cytokines, growth factors, proteolytic enzymes, and scavenger receptors. By doing so, they play a central role in regulating several physiological and pathological processes. These include: (1) Tissue homeostasis, e.g., removal of dead cells, senescent erythrocytes and cellular

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debris, as well as remodeling of extra-cellular matrix (ECM) components; (2) Developmental processes such as tissue morphogenesis, e.g., vascular and neuronal patterning and epithelial branching morphogenesis; (3) Adult tissue remodeling, e.g., in the bone and endometrium; (4) Tissue/organ healing and regeneration, e.g., wound repair and scar-tissue formation; (5) Pathological conditions, e.g., chronic inflammation and tumor growth (Gordon and Martinez 2010; Mantovani et al. 2013; Murray and Wynn 2011; Pollard 2009; Wynn et al. 2013). Of note, angiogenesis occurs in many of the aforementioned physiological or pathological processes.

It has long been recognized that activated monocytes/macrophages stimulate angiogenesis, an observation that dates back to the 1970s (Koch et al. 1986; Polverini et al. 1977; Sidky and Auerbach 1975; Squadrito and De Palma 2011; Sunderkötter et al. 1994). One of the initial hints that these cells have proangiogenic activity was provided by Polverini and colleagues, who showed that activated macrophages inoculated in the cornea of guinea pigs induced robust vascular proliferations. This suggested that macrophages are an important source of proangiogenic signals (Polverini et al. 1977). More recent studies have provided compelling evidence that activated macrophages are an important source of multiple cytokines and growth factors that modulate endothelial cell (EC) activation, proliferation, and/or survival (Squadrito and De Palma 2011). Moreover, macrophages express membrane-bound or secreted proteases that remodel the ECM and liberate matrix-bound proangiogenic growth factors, thus enabling EC activation, migration, and directional vascular growth (Baer et al. 2013; Squadrito and De Palma 2011). The proangiogenic functions of macrophages have been actively studied in the context of cancer. In tumors, high numbers of macrophages often correlate with increased angiogenesis (Leek and Lewis 1999; Leek et al. 1996). Bone-marrow or spleen derived monocytes are recruited to tumors, where they rapidly differentiate into mature and short-lived tumor-associated macrophages (TAMs). Whereas TAMs can exert either tumor-promoting or suppressive functions depending on their activation state and the cytokine milieu present in distinct microenvironments, studies in mouse models of cancer show that TAMs are largely protumoral, a feature that depends—at least in part—on their ability to sustain tumor angiogenesis (Coffelt et al. 2009; De Palma and Lewis 2013; Qian et al. 2010; Squadrito and De Palma 2011). In addition to pathological conditions characterized by dysregulated (excessive) angiogenesis (such as cancer and diabetic retinopathy), there is also compelling evidence for macrophages to regulate physiological angiogenesis in developmental processes such as retinal vascularization. In this context, macrophages finely tune vascular morphogenesis by conveying both pro- and antiangiogenic signals (Baer et al. 2013).

In this chapter, we discuss published studies that attest to the ability of macrophages to regulate angiogenesis (the formation of new blood vessels), arteriogenesis (the enlargement of pre-existing arteriolar connections), and lymphangiogenesis (the formation of new lymphatic vessels), both in developmental and post-natal life. This book chapter is based in part on recent review articles published by one of the authors (M.D.P.) and his colleagues on this topic (Baer et al. 2013; Nucera et al. 2011; Squadrito and De Palma 2011).

7.2 Macrophage Modulation of Angiogenesis

Several studies have shown that macrophages are implicated in vascular morphogenesis during development and post-natal organ remodeling. These studies have shown that, in addition to their expression of proangiogenic and tissue-remodeling factors, macrophages assist blood vessel growth and anastomosis via cell-to-cell contacts with ECs (Fantin et al. 2010; Rymo et al. 2011). Macrophages also enable vascular patterning and remodeling by secreting proapoptotic or antiangiogenic factors that regress "temporary" blood vessels via EC apoptosis (Lobov et al. 2005; Rao et al. 2007; Stefater et al. 2011).

The de novo formation of embryonic blood vessels—a process called vasculogenesis—involves the differentiation and migration of mesoderm-derived endothelial progenitor cells (EPCs), which create a primitive vascular network. Later in development, new blood vessels arise from pre-existing ones through angiogenesis in response to local tissue-derived signals (Herbert and Stainier 2011). These angiogenic cues lead to disruption of endothelial cell–cell junctions and activation of proteases that degrade the surrounding basement membranes. As such, ECs acquire a motile phenotype that facilitates the initiation of vessel sprouting. Sprouting angiogenesis requires the hierarchical organization of sprouting vessels into leading "tip" and trailing "stalk" ECs. Endothelial tip cells lead sprouting vessels, extend filopodia, and migrate in response to gradients of secreted proangiogenic ligands, namely VEGFA. On the other hand, adjacent stalk cells trail tip cells, generate the trunk of new vessels, and maintain connectivity with parental vessels (Herbert et al. 2012). Once initiated, EC sprouting continues in a directional manner until the endothelial tip cells at the forefront connect and fuse with adjacent vessels (anastomosis). Tight endothelial cell–cell junctions are subsequently formed between the newly formed and the pre-existing blood vessels, thus enabling blood flow through a continuous vessel lumen (Herbert and Stainier 2011).

Recent studies have highlighted the involvement of several cell types in the aforementioned process. Of note, macrophages promote vessel sprouting by secreting or liberating ECM-embedded proangiogenic factors; furthermore, they facilitate the fusion of vascular sprouts to enable anastomosis (Baer et al. 2013). Other accessory cells, such as pericytes and vascular smooth muscle cells (SMCs), play critical roles in the subsequent maturation and stabilization of the vasculature (Adams and Alitalo 2007; Jain 2003).

7.2.1 Retinal and Hyaloid Vessel Morphogenesis

The morphogenesis of both retinal and hyaloid vessels requires the participation of macrophages (Baer et al. 2013).

The retinal vasculature forms post-natally at the optic nerve head with the radial expansion of a superficial vascular plexus, which subsequently projects vertical

sprouts into the retina. These sprouts expand radially inside the retina to form the deep and intermediate vascular plexuses (Baer et al. 2013). Kubota et al. (2009) showed that colony-stimulating factor-1 (*Csf1*)-deficient mice (also known as *Csf1*$^{op/op}$ mice), which lack macrophages, display reduced branching of the superficial vascular plexus at post-natal day 2. This finding indicates that macrophages are important for retinal angiogenesis during the first week of the mouse life. Indeed, the vascular defects observed in *Csf1*$^{op/op}$ mice subsided at later stages of post-natal development, and the retinal vasculature of the mutant mice became comparable to that of wild-type mice at 3 months after birth (Baer et al. 2013; Kubota et al. 2009).

Rymo and colleagues provided further evidence that macrophages have a supportive role for retinal angiogenesis (Rymo et al. 2011). These authors showed that macrophages associate with endothelial tip cells at the forefront of the growing superficial vascular plexus of the mouse retina. Macrophages were found to physically interact with adjacent endothelial tip cells, a process that may facilitate anastomosis of vascular sprouts (see below). In agreement with findings of Kubota et al. (2009), the developing retinas of *Csf1*$^{op/op}$ mice displayed fewer vascular sprouts and a less complex vascular network (Rymo et al. 2011). Recently, the NOTCH pathway has been proposed to regulate macrophage–EC interactions during anastomosis of retinal vascular sprouts (Box 7.1).

Macrophages interact with ECs and modulate angiogenesis also in the deep layers of the post-natal retina. Interestingly, macrophages play an important role in limiting excessive vessel branching in this context (Stefater et al. 2011). Indeed, Stefater and colleagues have shown that retinal macrophages produce WNT ligands that negatively regulate blood vessel branching. Macrophage deletion of the WNT ligand transporter, *Wntless*, results in plethoric angiogenesis in the deep retinal layer. Mechanistically, non-canonical WNT signaling in the macrophages stimulates them to secrete soluble VEGFR1 (sFLT1), a molecule that sequesters VEGFA and hence limits its bioavailability (Eubank et al. 2004; Stefater et al. 2011). Thus, retinal macrophages may regulate vessel branching and network formation through both pro- and antiangiogenic cues. This is consistent with the notion that retinal angiogenesis is subject to extensive remodeling and pruning following the formation of the first vascular plexuses (Baer et al. 2013; Saint-Geniez and D'Amore 2004).

Insight into the functions of macrophages during vascular development was also provided by studies on the hyaloid vessels, a temporary vascular network that supports the lens during development of the eye (Saint-Geniez and D'Amore 2004). Indeed, it was found that the hyaloid vessels can develop normally but fail to undergo regression in *PU.1*$^{-/-}$ mice, which lack macrophages (Lobov et al. 2005). During hyaloid vessel regression, perivascular macrophages release WNT7B, a molecule that induces cell-cycle progression and sensitizes ECs to angiopoietin-2 (ANG2)-induced apoptosis (Lobov et al. 2005; Rao et al. 2007). Thus, although macrophages are dispensable for EC proliferation during the formation of hyaloids vessels, they seem to play an important role during their regression phase (Lobov et al. 2005; Rao et al. 2007).

Together, the aforementioned data support the notion that, in the developing eye, perivascular macrophages finely tune angiogenesis both by promoting blood vessel branching and anastomosis—a process that may entail macrophage–endothelial cell-to-cell contacts (Kubota et al. 2009; Rymo et al. 2011)—and by limiting the formation of excessive vessel branches—a process that is tightly controlled by WNT, ANG2, and sFLT1 signaling and EC apoptosis (Lobov et al. 2005; Rao et al. 2007; Stefater et al. 2011).

7.2.2 Hindbrain Angiogenesis

In the developing hindbrain, macrophages associate with sites of active vascular sprouting, where they function as modulators but not inducers of angiogenesis (Baer et al. 2013; Nucera et al. 2011). In wild-type mouse and zebrafish embryos, yolk sac-derived macrophages are often seen in close association with sprouting blood vessels (Fantin et al. 2010; Pucci et al. 2009). For example, macrophages were shown to accumulate in the subventricular zone of the hindbrain between 10 and 11.5 days post-coitum, a time point when new blood vessels branch to form the subventricular vascular plexus (Fantin et al. 2010). Once the subventricular vascular plexus had formed, macrophages decreased in this anatomical region and began to accumulate in the deeper brain layers, concomitant with the branching of radial arteries. These observations suggest that the branching of new blood vessels requires the participation of macrophages. To further address the role of these cells in vascular patterning, Fantin and colleagues used macrophage-deficient, *PU.1*$^{-/-}$ mouse embryos (Fantin et al. 2010). They found that the hindbrains of *PU.1*-mutant and wild-type mice expressed similar VEGFA levels, suggesting that macrophages do not provide a major source of VEGFA during brain development. Moreover, *PU.1*$^{-/-}$ hindbrains had normal numbers of vascular sprouts, but displayed significantly fewer intersections among the blood vessels. Importantly, macrophages frequently "bridged" adjacent vascular sprouts in wild-type embryos. Together, these data argue that macrophages support anastomosis of sprouting blood vessels through physical contacts with the endothelial-tip cells, a process that occurs downstream to VEGF-induced vessel sprouting and that is required for the formation of complex vascular networks (Fantin et al. 2010). Of note, the fetal macrophages that "bridge" neighboring endothelial-tip cells express the ANG receptor, TIE2, and the VEGF co-receptor, neuropilin-1 (NRP1), thus they resemble phenotypically the perivascular macrophages that support tumor angiogenesis (see below). While it is conceivable that "bridging" macrophages aid the fusion of vascular sprouts by increasing their proximity, the molecular signals that regulate macrophage–EC interactions during vascular anastomosis are still poorly understood (see Box 7.1 below for an account on the putative role of NOTCH signaling in this process).

7.2.3 Retinopathies

Angiogenesis occurs only in a few physiological conditions during adult life (Carmeliet 2003; Ferrara and Kerbel 2005). However, in both physiopathological (e.g., wound healing) and pathological (e.g., diabetic retinopathy and cancer) conditions, a cascade of molecular and cellular events primed by hypoxia, inflammation, and/or tissue damage, may lead to the activation of angiogenetic programs.

Ischemic retinopathies, such as those associated with diabetes, are the leading cause of visual loss in individuals under the age of 55. Age-related macular degeneration (AMD) is a severe, chronic form of ischemic retinopathy. Vision loss in these individuals occurs as a result of abnormalities in the retinal vasculature, leading to retinal edema, hemorrhage, gliosis (scarring), and/or neovascularization and, in some cases, tractional retinal detachment (Marchetti et al. 2011). Macrophages are thought to stimulate aberrant vascular growth in some of these pathological conditions, a process that is called choroidal neovascularization (CNV) when the appearance of new blood vessels occurs underneath the retina. Mouse models of CNV are generally obtained by laser-mediated injury of the retinal pigment epithelium.

Activated macrophages and microglia were associated with vessel growth in an explant model of human retina tissue (Knott et al. 1999). Further studies revealed that macrophage accumulation at sites of CNV was dependent on blood-derived monocytes rather than recruitment of resident microglia (Caicedo et al. 2005). By using laser-induced CNV and transplantation of GFP-labeled bone marrow (BM) cells, these authors observed that infiltrating, pan-macrophage marker+ (F4/80+) cells were mostly GFP+ (hence BM-derived) and associated closely with the blood vessels and activated Muller cells. Also, the density of resident (non-BM derived) microglia did not increase under CNV compared to wild-type mice (Caicedo et al. 2005). Several independent studies have then shown that these CNV-associated macrophages promote CNV. Macrophage depletion by clodronate liposomes attenuated CNV in mouse models (Espinosa-Heidmann et al. 2003; Sakurai et al. 2003). Similar results were obtained by using CCR2-deficient mice or wild-type mice treated with specific CCR2 inhibitors (the CCL2/CCR2 axis is an important regulator of monocyte/macrophage recruitment to inflamed tissues). For example, Chen and colleagues showed that while the density of infiltrating macrophages increases during disease progression in wild-type mice, genetic *Ccr2* deletion impairs macrophage recruitment to the injured retina and reduces the occurrence of neovascular membranes (Chen et al. 2012a). Similarly, a CCR2 antagonist limited macrophage infiltration in the laser-treated retina and prevented development of CNV; of note, macrophage blockade could also regress established neovessels (Xie et al. 2011).

As shown in developmental eye vascularization (see above), macrophages play multifaceted roles during pathological retinal vascularization. For example, Apte and colleagues have shown that interleukin (IL)-10-deficient ($IL10^{-/-}$) mice, which present exacerbated inflammation in response to inflammatory stimuli and increased inflammatory-macrophage infiltration in CNV, nonetheless display less severe CNV compared to wild-type (Apte et al. 2006). Furthermore, blocking macrophages by

anti-CD11b antibodies increased CNV in *IL10*$^{-/-}$ mice, whereas the direct injection of macrophages markedly reduced CNV in wild-type mice. CNV-associated macrophages were also found to express Fas-ligand (CD95L), which enabled killing of CD95-expressing choroidal vessels (Apte et al. 2006). These provocative observations suggest that CNV-associated macrophages can be antiangiogenic, at least in *IL10*$^{-/-}$ mice. While these results (Apte et al. 2006) may seem to conflict with the majority of the reports, which proposed a proangiogenic function for CNV-associated macrophages (Chen et al. 2012a; Espinosa-Heidmann et al. 2003; Sakurai et al. 2003; Xie et al. 2011), it is likely that specific experimental conditions (e.g., IL-10 deficient vs. proficient mice; genetic vs. pharmacological macrophage depletion; severity of laser-induced retinal injury and extent of local inflammation; extent of CD95 expression by neovascular sprouts; etc.) may influence the activation state and effector functions of CNV-associated macrophages, hence tilting their ability to either promote or inhibit angiogenesis in this disease model. Of note, IL-10 is known to suppress the proinflammatory and cytotoxic functions of macrophages (Biswas and Mantovani 2010).

Box 7.1 Role of NOTCH Signaling in Macrophage–EC Cross-Talk

The NOTCH pathway is a conserved ligand–receptor signaling mechanism that modulates cell fate decisions, differentiation, proliferation, and apoptosis (Dufraine et al. 2008; Greenwald 1998). Notch signaling depends on cell-contact-dependent interactions. The NOTCH receptors (NOTCH1-4) are transmembrane proteins consisting of an extracellular domain that is responsible for ligand interaction; a transmembrane domain that is involved in receptor activation; and an intracellular signaling domain. Notch signaling is activated upon binding of a ligand (JAGGED1 and 2, Delta-like ligand (DLL) 1, 3 and 4), which is expressed on the surface of neighboring cells, to the extracellular domain of the NOTCH receptor. This binding leads to a series of biochemical events and enzymatic cleavages that promote the release of the extracellular domain and the translocation to the nucleus of the intracellular domain, which regulates gene transcription (Dufraine et al. 2008). Several studies have shown that NOTCH signaling is of crucial importance during the development of the vascular system in both embryonic and postnatal angiogenesis. During angiogenesis, DLL4/NOTCH1 signaling is involved in modulating EC decision, i.e., whether an EC will acquire a leading ("tip") or growing ("stalk") phenotype (Alva and Iruela-Arispe 2004; Lobov et al. 2007; Takeshita et al. 2007; Kofler et al. 2011).

Macrophages have been reported to express both NOTCH receptors and some of their ligands (Singh et al. 2000). NOTCH signaling in macrophages may regulate their gene expression and proinflammatory cytokine production

(continued)

Box 7.1 (continued)

(Monsalve et al. 2006, 2009; Outtz et al. 2010; Palaga et al. 2008). Furthermore, direct contacts between macrophages and ECs may activate NOTCH signaling in the macrophages and regulate the function of angiogenic ECs. Indeed, NOTCH1 was found to regulate macrophage recruitment and activity during anastomosis of developing retinal vessels (Outtz et al. 2011). Whereas retinal vascular density and tip cell sprouting were not altered in mice with myeloid-cell specific deletion of *Notch1*, macrophage accumulation was significantly decreased at both vascular branch-points and the leading edge of the vascular plexus. Intriguingly, the authors observed an increased frequency of elongated EC sprouts that did not form anastomoses with neighboring vascular sprouts in mice with *Notch1*-deficient myeloid cells (compared with control mice). In non-mutant mice, NOTCH1-proficient macrophages were shown to bind to DLL4+ endothelial tip cells undergoing anastomosis (Outtz et al. 2011). These data suggest that NOTCH1 is important for the localization of macrophages at the vascular front where active sprouting and EC anastomosis occur. Nevertheless, the exact mechanisms by which NOTCH signaling regulates macrophage-mediated vessel branching and anastomosis remain to be elucidated. Of note, NOTCH1 was also shown to induce VEGFR1 expression in macrophages (Outtz et al. 2010). Given the important role of VEGFA as a macrophage chemoattractant in tissues undergoing angiogenesis (Barleon et al. 1996; Cursiefen et al. 2004), this observation suggests that NOTCH signaling may play a role in the regulation of monocyte/macrophage recruitment to angiogenic (VEGF-expressing) tissues.

Macrophages may also modulate NOTCH signaling in ECs. Tammela and colleagues have indeed shown that macrophage-derived VEGFC modulates EC sprouting by activating VEGFR3 in endothelial tip cells and reinforcing NOTCH signaling. This promoted the tip-to-stalk phenotypic conversion of ECs at the fusion points of retinal vessel sprouts (Tammela et al. 2011). In addition, genetic inactivation of VEGFR3 in ECs resulted in increased numbers of endothelial tip cells, which closely resembled loss of NOTCH signaling (Tammela et al. 2011). Based on all the above, it is likely that macrophage–EC contacts activate NOTCH signaling in either cell type to regulate vascular morphogenesis.

7.2.4 Skin Wound Angiogenesis

Skin wound healing is a complex biological process that occurs frequently during adulthood and involves the interplay among different cell types, including keratinocytes, fibroblasts, and inflammatory cells (Martin 1997; Nucera et al. 2011; Rodero and Khosrotehrani 2010). Early studies suggested that macrophages and neutrophils

play a crucial role during skin wound healing (Leibovich and Ross 1975). Macrophages orchestrate the skin repair process by acting both as phagocytes (to clear dead cells and cellular debris) and as a source of growth factors (e.g., proangiogenic factors; epithelial-cell growth factors; etc.).

Macrophages participate in the diverse phases of skin repair: inflammation; tissue formation; and tissue maturation (Lucas et al. 2010). By crossing transgenic mice that express the CRE recombinase from the lysozyme M (LysM) promoter, with transgenic mice that express the human diphtheria toxin (DT) receptor upon CRE-mediated recombination, the authors generated mice in which depletion of macrophages and other myeloid cells can be induced during specific stages of the repair response. Depletion of myeloid cells during the early stage of the repair response (inflammatory phase) significantly reduced the formation of vascularized granulation tissue, impaired epithelialization, and resulted in minimized scar formation (Lucas et al. 2010). Furthermore, depletion of macrophages during the subsequent phase of the repair response (tissue formation) caused severe hemorrhage in the wound tissue and prevented wound closure. On the other hand, macrophage depletion during the late stage of repair (tissue maturation) did not significantly impact the outcome of the repair response (Lucas et al. 2010). These observations suggest that macrophages exert important functions during the early phases of skin repair, when they appear to play an important function in promoting angiogenesis in the granulation tissue during the inflammatory phase and vascular growth and stabilization in the tissue formation phase (Lucas et al. 2010; Nucera et al. 2011).

Distinct "types" of wound healing macrophages have been described to date (Nucera et al. 2011; Rodero and Khosrotehrani 2010). Okuno et al. (2011) showed that a sizable fraction of the BM-derived cells that infiltrate either acute (dorsal excisional ear punch) or chronic (decubitus ulcer) skin wounds are macrophages. Most of these wound-healing macrophages display a "pro-tissue remodeling" phenotype, which is characterized by high expression of scavenger receptors and relatively low levels of proinflammatory cytokines, such as nitric oxide synthase-2 (NOS2) and IL-6. Consistent with the location of proangiogenic macrophages in tumors (Baer et al. 2013), the authors found that the majority of these wound-healing macrophages were present in perivascular areas at the wound healing site (Okuno et al. 2011).

Recruitment of macrophages to healing wounds may be regulated by different chemoattractants, such as CCL2, macrophage inflammatory protein-1alpha (MIP1α), CXCL12 (also known as SDF1), and CSF1 (Nucera et al. 2011; Wu et al. 2010). Okuno et al. (2011) showed that CSF1 is up-regulated at early stages during wound healing and plays an important role in recruiting macrophages. Blocking CSF1 or CSF1R dramatically reduced the macrophage infiltrate. Moreover, *Csf-1*$^{op/op}$ mice had delayed wound healing, which correlated with decreased vascular density. Interestingly, the overall amounts of classic proangiogenic factors produced at the wounded site did not differ between *Csf1*$^{op/op}$ and wild-type mice, but matrix-metalloproteinases (MMPs) were significantly reduced. Together, these observations suggest that wound healing macrophages exert their proangiogenic functions—at least in part—by physically associating with sprouting blood vessels and facilitating ECM remodeling via MMP activity (Nucera et al. 2011; Okuno et al. 2011).

As observed during developmental and pathological retinal vascularization, macrophages may also negatively regulate angiogenesis in healing wounds. Indeed, Stefater and colleagues found that macrophages may use a WNT–Calcineurin–NFAT–sFLT1 pathway to limit wound angiogenesis in mice (Stefater et al. 2013). Genetic deletion of either *Wintless*, *Cnb1* (a subunit of the calcineurin–NFAT complex) or *Flt1* in macrophages (as well as subsets of lymphocytes and neutrophils) increased wound angiogenesis and accelerated repair. As discussed above, macrophages employ a similar mechanism to control excessive angiogenesis during retinal vascularization (Stefater et al. 2011). Whereas these observations require further validation by alternative genetic approaches, they are somewhat consistent with an earlier report showing that macrophage-deficient *PU.1*$^{-/-}$ mice are able to repair skin wounds with similar time course to wild-type siblings (Martin et al. 2003). The evidence for wound-healing macrophages to both enhance and limit wound angiogenesis attests to the complex and multifaceted functions of these cells in angiogenesis. Indeed, macrophage pro- vs antiangiogenic activity may be modulated by specific microenvironmental cues, hence be context-dependent (Lucas et al. 2010; Nucera et al. 2011). In this regard, Sindrilaru and colleagues showed that human chronic venous ulcers contain increased proportions of macrophages that express a proinflammatory phenotype (e.g., elevated NOS2 and tumor-necrosis factor-α [TNFα]) than in healing wounds (Sindrilaru et al. 2011). As discussed below, proinflammatory (or M1-like) macrophages sustain tissue inflammation and actively phagocytose dead cells and cellular debris. The authors found that elevated iron deposits in chronic wounds, and their uptake by macrophages, impair macrophage programming from the proinflammatory to the "wound-healing"/"pro-tissue-remodeling" (or M2-like) phenotype, a phenotypic switch that instead occurs in healing wounds (Sindrilaru et al. 2011). This study therefore suggests that proinflammatory macrophages likely prevail over wound-healing macrophages in early (inflammatory) or chronic wounds, where they may prevent infection and facilitate the clearance of necrotic tissue (Sindrilaru et al. 2011). It is currently unclear whether "wound-healing" macrophages derive from inflammatory macrophages (via in situ programming) or from a distinct monocyte precursor (Nucera et al. 2011).

7.2.5 Tumor Angiogenesis

During tumor growth, hypoxic microenvironments stimulate a potent proangiogenic response (Weis and Cheresh 2011). This is exemplified by the dramatic enhancement of vascular density that occurs in tumors during the benign-to-malignant transition, a process referred to as the "angiogenic switch" (Hanahan et al. 1996). The quantity and variety of proangiogenic growth factors expressed in growing tumors generally induce a highly chaotic, hypertrophic, and ostensibly dysfunctional tumor-associated vasculature (McDonald and Choyke 2003). In addition to cancer cells, research performed over the past 15 years has implicated different hematopoietic cell types, including macrophages, mast cells, and neutrophils, as potent

proangiogenic cells (Coffelt et al. 2010; Hanahan and Coussens 2012; De Palma and Naldini 2006; De Palma and Coussens 2008; Qian and Pollard 2010; Squadrito and De Palma 2011).

As outlined in Box 7.2 below, monocytes/macrophages are pleiotropic innate immune cells that are avidly recruited to tumors in response to cancer cell death, hypoxia, and tumor-secreted chemoattractants (De Palma and Lewis 2013; Qian

Box 7.2 Origins of Tumor-Associated Macrophages

How are TAMs recruited to the tumors? TAMs derive from circulating monocytes (De Palma et al. 2003), which are recruited to the tumor site by chemokines, cytokines, and growth factors produced by both cancer and stromal cells. These include CCL2 (also known as MCP1), CCL5, CCL7, CCL8, CXCL12 (also known as SDF1), CSF1, transforming growth factor-β (TGF-β), VEGF, placental growth factor (PlGF), platelet-derived growth factor (PDGF), and others (Balkwill 2004; Mantovani and Sica 2010; Murdoch et al. 2008; Squadrito and De Palma 2011). Many of these chemotactic molecules are under the control of transcriptional factors, such as NF-kB (Karashima et al. 2003) and HIF1α (Liao and Johnson 2007), which are activated in cancer and stromal cells by inflammatory mediators and hypoxia, respectively. Hypoxia-induced chemokines are a major determinant for macrophage recruitment to tumors. Once recruited to hypoxic tumor areas, activation of HIF1α in TAMs promotes their retention at these sites, possibly as a consequence of impaired chemotactic signaling (Murdoch et al. 2008). HIF1α activation in TAMs also primes their proangiogenic programming and ability to stimulate angiogenesis (Du et al. 2008; Leek and Lewis 1999; Murdoch et al. 2008). It should be noted, however, that TAMs accumulate in various tumor microenvironments, including nonhypoxic perivascular areas (Mazzieri et al. 2011; De Palma et al. 2005; Squadrito and De Palma 2011).

Macrophage accumulation within tumors is largely sustained from hematopoietic progenitor cells (HPCs), which proliferate and differentiate into promonocytes in the bone marrow (BM), before they are shed in the blood circulation as monocytes. These monocytes can then undergo final differentiation into TAMs as they extravasate in the tumors. Indeed, bone marrow (BM) transplantation experiments using gene-marked HPCs showed that macrophages are recruited to mouse tumors from BM-derived circulating monocytes (De Palma et al. 2003). Likewise, BM-derived HPCs may accumulate and expand in the spleen, which can become an important reservoir of monocyte production. These monocytes can physically relocate from the spleen to the tumor stroma, a process that is dependent—at least in part—on CCL2/CCR2 signaling (Cortez-Retamozo et al. 2012). Although monocytes are regarded as TAM precursors, it is still unclear which of the distinct

(continued)

Box 7.2 (continued)

circulating monocyte subtypes primarily contributes to the pool of TAMs observed in mouse tumors. Indeed, circulating monocytes can be divided into at least three phenotypically and functionally distinct subsets, which are generally referred to as classical (or inflammatory), intermediate, and non-classical (or resident) monocytes (Geissmann et al. 2008; Ziegler-Heitbrock et al. 2010). The monocytic origin of TAMs is still controversial as both the classical (Movahedi et al. 2010) and nonclassical (MacDonald et al. 2010) monocytes were shown to be prospective TAM precursors. Furthermore, a recent study showed that while nonclassical monocytes largely contribute to TAMs residing in "primary" mammary tumors, classical monocytes are preferentially recruited to their pulmonary metastases, thus suggesting that the origin of TAMs may differ in primary versus metastatic tumors (Qian et al. 2011). The origin of human TAMs is currently unknown.

There is also increasing evidence for some tissue-resident macrophages (e.g., brain microglia and liver Kuppfer cells) to be maintained through local proliferation under steady state conditions (Yona and Jung 2010). Furthermore, recent studies showed that local proliferation can contribute to macrophage accumulation and expansion during acute inflammation (Jenkins et al. 2011). Hence, it is plausible that local proliferation may contribute, at least to some extent, to macrophage accumulation in tumors. Indeed, CSF1 secreted by cancer or stromal cells, as well as T-cell derived interleukin (IL)-4, can both support TAM proliferation (Galdiero et al. 2012; Jenkins et al. 2011). However, TAM proliferation/mitosis (Cortez-Retamozo et al. 2012; Galdiero et al.2012; Movahedi et al. 2010) and colony-forming cells (Pucci et al. 2009) are rare in mouse tumors, making it unlikely that local proliferation of myeloid or monocyte progenitors significantly contributes to macrophage turnover in tumors.

and Pollard 2010). Once in tumors, TAMs appear to foster rather than contrast tumor growth and progression. The multifaceted protumoral (e.g., proinvasive and immunosuppressive) functions of TAMs have been discussed elsewhere (Mantovani and Sica 2010; De Palma and Lewis 2013; Qian and Pollard 2010). Among these, TAMs convey proangiogenic signals that sustain robust tumor vascularization (Fig. 7.1). Intriguingly, the proangiogenic and tissue-remodeling functions of TAMs are remarkably similar to those of wound-healing macrophages (Baer et al. 2013; Nucera et al. 2011; Squadrito and De Palma 2011).

In 1996, Leek and colleagues reported a positive correlation between the abundance of TAMs and the vascular grade of human breast cancers (Leek et al. 1996). Subsequent studies confirmed this association in other human tumor types (De Palma and Lewis 2013; Qian and Pollard 2010). During the past decade, several studies employing mouse tumor models documented the proangiogenic activity of TAMs, or subpopulations thereof. For example, genetically depleting perivascular

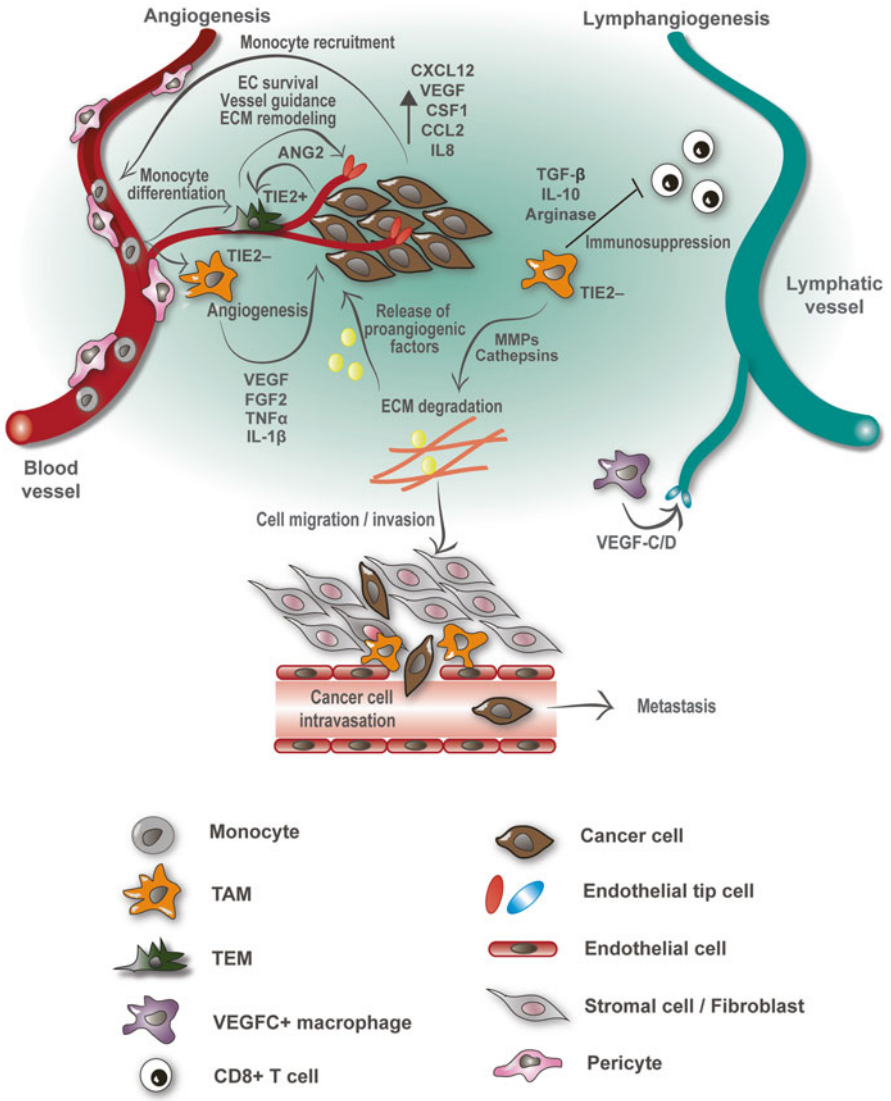

Fig. 7.1 Regulation of tumor angiogenesis and lymphangiogenesis by macrophages. TAMs promote tumor progression by facilitating angiogenesis (*top left*), lymphangiogenesis (*top right*), immunosuppression (*top right*), and cancer cell invasion and metastasis (*middle*). Studies in mouse models of cancer have established the importance of macrophages, or subsets thereof, in the regulation of tumor angiogenesis. Monocytes are recruited to tumors from the circulation by tumor-derived factors such as VEGFA, CCL2, CSF1, CXCL12, IL8, and others. Once recruited, monocytes differentiate into TAMs. TAMs found in tumors display significant phenotypic and functional heterogeneity. TAMs regulate tumor angiogenesis by both secreting proangiogenic growth factors (e.g., VEGFA, FGF2, TNFα, IL1β, and others) and remodeling the ECM, thus enabling the mobilization of proangiogenic growth factors embedded therein. Perivascular TAMs, which express the ANG receptor TIE2 (TEMs), may respond to EC-derived ANG2 and promote angiogenesis also by enhancing EC survival and assisting the growth of sprouting blood vessels via cell-to-cell contacts

TAMs (also known as TIE2-expressing macrophages (TEMs); see below) using a conditional suicide-gene approach (Herpes simplex virus/thymidine kinase [HSV-TK]/GCV) was sufficient to inhibit tumor vascularization and growth in several tumor models, including human gliomas growing orthotopically in the mouse brain. Furthermore, co-injection of TEMs with cancer cells enhanced angiogenesis in incipient tumors (De Palma et al. 2003, 2005). Likewise, crossing mice that develop oncogene-induced mammary tumors (MMTV-PyMT) with macrophage-deficient *Csf1*$^{op/op}$ mice delayed tumor progression and partially inhibited tumor angiogenesis in MMTV-PyMT/*Csf1*$^{op/op}$ mice. Conversely, the genetic overexpression of *Csf1* in the mammary epithelium of MMTV-PyMT mice resulted in the premature accumulation of macrophages into hyperplastic lesions and accelerated the angiogenic switch and tumor progression (Lin et al. 2006a). Similar results were obtained when TAMs were depleted from tumors (or otherwise inhibited), for example, by using pharmacological approaches like neutralizing anti-CSF1/CSF1R antibodies or liposome-encapsulated clodronate, a bisphosphonate that kills macrophages upon phagocytosis (Giraudo et al. 2004; Priceman et al. 2009; Zeisberger et al. 2006; Zhang et al. 2010). Together, the aforementioned studies have provided compelling evidence for TAMs to support tumor angiogenesis, at least in these rapidly growing and aggressive mouse tumor models.

TAMs are thought to stimulate sprouting of pre-existing blood vessels via three main mechanisms:

1. Secretion of classical proangiogenic growth factors and inflammatory mediators. These can directly stimulate EC proliferation, growth, or survival;
2. Expression and/or secretion of proteases and other matrix-remodeling enzymes. These can break-down the ECM and liberate proangiogenic growth factors embedded and hence sequestered within. ECM remodeling may also facilitate the navigation of vascular sprouts through the dense ECM;
3. Physical association with ECs through cell-to-cell contacts. This may enhance EC survival and migration, and also facilitate vascular anastomosis (shown in fetal development; see above).

TAMs (or activated macrophages) have been reported to express and secrete a large assortment of proangiogenic growth factors, such as VEGFs, PlGF, fibroblast growth factor (FGF)-2 and TNFα; proangiogenic interleukins (e.g., IL-8/CXCL8), and chemokines (e.g., CXCL12); and several vascular-guidance molecules, such as semaphorin (SEMA)-4D, 3B, and 3A. All these molecules bind to cognate receptors expressed on the surface of ECs, thus stimulating their proliferation and/or survival (Coffelt et al. 2009; Dirkx et al. 2006; Murdoch et al. 2008; Squadrito and De Palma 2011; Sunderkötter et al. 1994). Furthermore, it is likely that TAMs—similar to other cell types—secrete microvesicles (MVs) called exosomes, which have the ability to fuse with, and transfer their cargo of proteins and RNAs to, "acceptor" cells (Baer et al. 2013). It is, however, currently unknown whether TAM-derived MVs can fuse with ECs in tumors, and if potential transfer of their cargo can foster angiogenic programming of the tumor blood vessels. Further studies are warranted that investigate this intriguing scenario.

Several proangiogenic growth factors, such as VEGF, FGF2, and TNF-α, are highly expressed in tumors. However, their bioavailability is limited, as they are either sequestered to the ECM or tethered to the cell membrane (De Palma and Coussens 2008). TAMs secrete multiple proteases that, via remodeling of the ECM, regulate the release of these factors and make them available for interaction with their receptors on ECs. Important TAM-derived proteases include MMPs and serine/cysteine proteinases (i.e., urokinase and cathepsins). Bergers and colleagues used a mouse model of oncogene-induced pancreatic insulinoma (RIP1-Tag2) to show that MMP9-expressing myeloid cells (comprising macrophages and neutrophils) are essential to liberate ECM-bound VEGFA and trigger the angiogenic switch in premalignant tumors (Bergers et al. 2000). Further to liberating sequestered growth factors, TAM-derived proteolytic enzymes may facilitate EC migration within the ECM. In the ischemic heart, monocytes/macrophages were found to "drill" tunnels in the ECM (via their secretion of metallo-elastase) that were subsequently endothelialized (Moldovan et al. 2000). These important observations suggest that macrophages may help digest the dense ECM and "pave the way" to sprouting blood vessels also in the context of tumors. In fact, the association of perivascular TAMs with newly formed tumor blood vessels (as observed on static tumor sections) may reflect a dynamic process of collective cell migration whereby TAMs develop provisional channels in the ECM that are subsequently colonized by vascular ECs.

As mentioned above, macrophages regulate vascular morphogenesis during embryonic development by establishing cell-to-cell contacts with ECs and "guiding" vascular anastomosis (Fantin et al. 2010; Rymo et al. 2011). Interestingly, direct macrophage–EC interactions that support angiogenesis have also been observed in tumors (Mazzieri et al. 2011; De Palma et al. 2005). Similar to embryonic/fetal macrophages, perivascular TAMs express the ANG receptor, TIE2 (Mazzieri et al. 2011; De Palma et al. 2005). As noted above, the specific depletion of these TEMs inhibits angiogenesis in mouse tumor models (De Palma et al. 2003, 2005). Because TEMs represent a minor proportion of all TAMs (De Palma and Naldini 2009), it is unlikely that their elimination in tumors depletes a critical source of classic proangiogenic growth factors, such as VEGFA. Furthermore, TIE2-negative TAMs, which mostly reside in tumor areas distant from the blood vessels (e.g., highly hypoxic or necrotic regions), express VEGFA to a greater extent than perivascular TEMs (Pucci et al. 2009). Thus, TEMs are likely to support tumor angiogenesis by alternative (i.e., VEGF-independent) mechanisms. Mazzieri and colleagues suggested that direct TEM–EC contacts are critical for TEM-mediated angiogenesis in tumors. In particular, they found that the ANG2/TIE2 axis modulates TEM–EC interactions during tumor angiogenesis (Mazzieri et al. 2011). This was shown by using a conditional knock-down strategy to silence *Tie2* expression in macrophages. TIE2 suppression in perivascular macrophages impeded their association with the blood vessels and decreased tumor vascularization in different mammary tumor models. Similar results were obtained by pharmacological blockade of ANG2, the main TIE2 ligand in tumors (Mazzieri et al. 2011; De Palma and Naldini 2011). The above findings provide preliminary evidence that TIE2-expressing ECs and TEMs interact via TIE2–ANG2–TIE2 complexes that form at their cell contacts.

Additional ligand–receptor pairs may regulate functional EC–macrophage contacts in tumors. For example, CXCL12, which is primarily secreted by perivascular stromal cells, may recruit CXCR4$^+$ monocytes/macrophages from the circulation and retain them in perivascular spaces (Baer et al. 2013; Grunewald et al. 2006). Of note, TEMs were shown to express CXCR4 to a higher extent than TIE2-negative TAMs (Welford et al. 2011), an observation that may suggest a role for EC-derived CXCL12 in recruiting TEMs to perivascular spaces. Taken together, the aforementioned studies suggest that TAMs (or subsets thereof, such as TEMs; see Box 7.3 for

Box 7.3 Macrophage Heterogeneity in Tumors: Role of Th1 and Th2 Cytokines in the Regulation of TAM's Proangiogenic Functions

Tissue macrophages are inflammatory cells that can display different activation states according to distinct signals present in the local microenvironment. As discussed by other authors of this book, two extremes of these activation states have been termed classical (or M1) and alternative (or M2) activation, in analogy to the T-helper (Th) type-1 (Th1) and Th2 classification of lymphocytes (Gordon and Martinez 2010; Gordon and Taylor 2005; Mantovani et al. 2002, 2009). Under cell culture conditions, inflammatory mediators such as lypo-polysaccharide (LPS), TNF-α or interferon-gamma (INF-γ) activate macrophages to acquire an M1-polarized phenotype. In response to such stimuli, M1-polarized macrophages increase their expression of proinflammatory or angiostatic cytokines (e.g., IL1β, TNF-α, IL12, CXCL19, CXCL10), as well as reactive oxygen and nitrogen species. On the other hand, macrophages acquire an M2 activation state after stimulation with the Th2 cytokines IL-4, IL-13, and IL-10. M2-polarized macrophages are characterized by higher expression of scavenger receptors, ECM-remodeling enzymes, and proangiogenic growth factors. Furthermore, they express lower proinflammatory (e.g., IL1β and TNF-α) and higher antiinflammatory (e.g., L-10 or TGF-β1) cytokine levels compared to M1-polarized macrophages (Gordon and Martinez 2010; Mantovani et al. 2002, 2009; Mills et al. 2000; De Palma and Lewis 2013; Sica and Mantovani 2012). M1 and M2-like macrophages (i.e., macrophages that acquire activation states that approximate those observed in vitro upon Th1/M1 or Th2/M2 stimulation) are also observed in vivo. Whereas M1-like macrophages are primarily found in inflamed tissues and display cytotoxic and antimicrobial activities, M2-like macrophages are observed in various patho(physio)logical conditions characterized by tissue remodeling or growth, such as would healing, atherosclerosis and cancer, and display proangiogenic, antiinflammatory and immunosuppressive functions (Galdiero et al. 2012; Gordon and Martinez 2010; Mantovani et al. 2002, 2013).

(continued)

Box 7.3 (continued)

Several tumor-derived factors can abate the cytotoxic and antitumoral activities of macrophages and induce them to acquire protumoral, immunosuppressive, and proangiogenic functions. Therefore, TAMs—similar to "wound-healing" macrophages—are regarded as prototypical M2-like macrophages (Mantovani et al. 2002). While in incipient tumors that arise at chronically inflamed sites TAMs may express an M1-like and hence cytotoxic phenotype that could contribute to T cell-mediated cancer elimination, in progressing tumors TAMs often express an M2-like phenotype characterized by low cytotoxicity, low antigen-presenting capacity, high immunosuppressing activity, and enhanced tissue-remodeling and proangiogenic properties (De Palma and Lewis 2013; Sica and Mantovani 2012). Some of these macrophage functions are directly promoted by Th2 cytokines like IL-4. For example, Gocheva and colleagues showed that IL-4 induces the expression of cathepsins B and S in macrophages both in vitro and in vivo (Gocheva et al. 2010). Cathepsins B and S have been shown to be important effectors for the tissue-remodeling and proangiogenic functions of M2-like TAMs in mice (Gocheva et al. 2010). Linde and colleagues showed that IL-4 signaling blockade using anti-IL-4 receptor-α (IL-4Rα) antibodies was sufficient to reprogram TAMs from an M2-like to an M1-like phenotype in transplant skin tumors (Linde et al. 2012; De Palma 2012). Moreover, genetic or pharmacological IL-4 signaling blockade blunted the proinvavise and prometastatic function of TAMs in mammary tumor models (DeNardo et al. 2009). A recent study implicated the mTOR pathway as a critical regulator of TAM's M2-like polarization. Indeed, inhibition of the mTOR repressor tuberous sclerosis complex-2 (TSC2) caused monocytes to differentiate into M2-like macrophages that released lower IL-12 and higher IL-10 and had greater proangiogenic activity in angiogenesis assays than their wild-type counterpart (Chen et al. 2012b). Yet, it should be noted that the M1 or M2 categories likely oversimplify the biology of TAMs, as different tumors (or distinct tumor microenvironments in the same tumor) may contain TAM subpopulations that display variegated activation states (De Palma and Lewis 2013; Sica and Mantovani 2012).

Gene expression profiling of TAMs using high-density oligonucleotide arrays showed that they are highly enriched in transcripts that encode proangiogenic molecules (e.g., VEGFA) and ECM-remodeling enzymes (e.g., ADAM8 and MMP14), which is consistent with a M2-like (or "wound-healing") phenotype (Ojalvo et al. 2009). Nevertheless, transcriptional profiling of distinct TAM subsets also suggested significant heterogeneity in individual mouse tumors (Movahedi et al. 2010; Pucci et al. 2009; Squadrito and De Palma 2011; Squadrito et al. 2012). For example, the gene expression profile of TEMs

(continued)

Box 7.3 (continued)

differs from that of TIE2-negative TAMs (Pucci et al. 2009). TEMs display enhanced expression of scavenger receptors such as hemoglobin/aptoglobin scavenger receptor (CD163), hyaluronan receptors (e.g., LYVE1), and mannose receptor (MRC1) compared to TIE2-negative TAMs. Furthermore, classic proinflammatory (e.g., IL-1β and TNF-α) and angiostatic cytokines (e.g., IL-12, CXCL9, and CXCL10) are downregulated in TEMs (Pucci et al. 2009). These findings suggest that TEMs display an extreme M2-like phenotype, which is consistent with their marked proangiogenic and protumoral functions (Mazzieri et al. 2011; De Palma et al. 2005; Pucci et al. 2009). Because TEMs are mostly perivascular in tumors and display a distinctly skewed M2-like phenotype, it is tempting to speculate that EC or vascular-derived factors enhance M2-like programming of this TAM subset in the perivascular microenvironment. Indeed, expression of the TIE2 receptor is upregulared in perivascular TAMs compared to circulating monocytes (De Palma et al. 2008; Pucci et al. 2009). Moreover, recent studies suggest that EC-derived CSF1 may have a role in promoting the "TEM" phenotype. Indeed, EC-derived CSF1 induced immature myeloid cells to differentiate into M2-like macrophages characterized by enhanced expression of VEGFA, TIE2, and MRC1 (He et al. 2012). Further studies are warranted to investigate if EC-derived CSF1 is indeed a critical modulator of TEM differentiation in tumors.

It should be noted that duly polarized macrophages can also exert angiostatic functions. This function has been mainly attributed to M1-like macrophages, as some molecules with angiostatic activity are highly expressed by classically activated macrophages (Dirkx et al. 2006; Mantovani et al. 2002). For example, M1-polarized macrophages express IL-12. Monocyte/macrophage-derived IL-12 activates T cell-dependent antitumor responses that can eradicate established tumors in mice (Dirkx et al. 2006; Tsung et al. 2002). IL-12 may also have angiostatic functions. These are unlikely to be mediated via direct effects on ECs, but rather involve INF-γ production by Th1 T and NK cells (Dirkx et al. 2006; Mitola et al. 2003). Indeed, the production of IL-12 by macrophages shifts $CD4^+$ T-cells toward the Th1 phenotype, which secrete IL-2 and IFN-γ and in turn enhance the proliferation and activation of $CD8^+$ cytotoxic T-cells, NK cells, and M1-like macrophages. In the latter cells, IFN-γ upregulates the production of CXCL10 and CXCL9, which have direct angiostatic properties (DeNardo et al. 2009; Dirkx et al. 2006; Mitola et al. 2003; Sgadari et al. 1996; Strasly et al. 2001). IL-18 has been shown to exert a similar activity to, and synergistic function with, IL-12 by initiating INF-γ production by Th1 T and NK cells, thus leading to inhibition of angiogenesis (Coughlin et al. 1998). Finally, macrophages secrete MMP12. This protease is associated with inhibition of angiogenesis, as it promotes the generation of angiostatin, a well-known angiostatic molecule, from plasminogen (Dong et al. 1997; Gorrin-Rivas et al. 2000). Consistent with this function, MMP-12 expression negatively correlates with blood vessel density in human tumors (Gorrin-Rivas et al. 2000).

an account of macrophage heterogeneity in tumors) may assist tumor angiogenesis also by establishing cell-to-cell contacts with ECs, a heterotypic cross-talk that may be regulated—at least in part—by ANG2/TIE2 and CXCL12/CXCR4 signaling.

Interestingly, He and colleagues recently proposed that activated ECs can function as a macrophage differentiation niche in angiogenic tissues (He et al. 2012). They found that ECs stimulate HPCs to directly differentiate into macrophages characterized by a proangiogenic phenotype. This process required cell-to-cell contacts between ECs and HPCs/macrophages and was dependent on EC-derived CSF1. EC-induced macrophages acquired a perivascular position and enhanced tumor angiogenesis and growth when co-injected with cancer cells in mice (He et al. 2012). Thus, functional EC–macrophage contacts may be bidirectional and support both angiogenesis and the differentiation of proangiogenic macrophages (Baer et al. 2013). Nevertheless, it should be noted that proangiogenic TAMs may not be exclusively perivascular. For instance, hypoxic TAMs may initiate angiogenesis by secreting high-levels of VEGFA. Consistent with this notion, whereas TAM accumulation was higher in poorly than highly vascularized tumor areas of human breast cancer specimens, the extent of macrophage infiltration correlated positively with the overall vascular grade of these tumors (Leek et al. 1996; Lewis et al. 2000).

7.2.6 Vascularization of Endometriotic Lesions

Endometriosis is a gynecological disease affecting 10–15 % of premenopausal women (Giudice 2010). It is characterized by the development and persistent growth of vascularized endometrial tissue outside of the uterine cavity, typically in the pelvis, and as such it is associated with abdominal pain, painful menstruation, and infertility (Becker and D'Amato 2007; Dunselman and Groothuis 2004). The development of this disease occurs gradually, in multiple steps. Initially, shed endometrial tissue attaches to the peritoneal wall. Subsequent steps include invasion of the underlying basement membrane and recruitment of novel blood vessels from the peritoneal vasculature (Becker and D'Amato 2007; Capobianco et al. 2011; Dunselman and Groothuis 2004).

As it has become apparent that angiogenesis plays a pivotal role in endometriosis progression, it is not surprising that many recent studies have highlighted the involvement of macrophages in the persistence and growth of ectopic endometrial lesions (Bacci et al. 2009; Capobianco et al. 2011; McLaren et al. 1996). However, the implication of other myeloid-derived cells, such as dendritic cells (DCs) and neutrophils, in inducing angiogenesis in endometriotic lesions has also been described (Fainaru et al. 2008; Lin et al. 2006b). In experimental models of endometriosis, early phases of lesion establishment are characterized by transient hypoxia, which results in the upregulation of HIF1α and downstream expression of VEGFA and chemokines that play a major role in the recruitment of macrophages (Becker et al. 2008; Capobianco 2013; Du et al. 2008; Machado et al. 2010). Once endometriosis is established, the cyclic and persistent death of endometrial cells

leads to the release of cell debris, erythrocytes, and heme-bound iron within the peritoneal cavity (Capobianco 2013). Prompted to phagocytose dead cells and cell debris, macrophages accumulate at the endometriotic lesions. These recruited macrophages are thought to activate a reparative/proangiogenic program that is required for the maintenance, growth, and spreading of endometriotic lesions (Bacci et al. 2009; Capobianco 2013). A role for NF-kB signaling in macrophages that accumulate in endometriotic lesions has been described. Indeed, angiogenesis in human endometriotic lesions was shown to be associated with the activation of the NF-kB pathway in macrophages, while inhibition of this pathway decreased lesion growth in vivo (Lousse et al. 2008).

A study of Bacci and colleagues has shown that macrophage recruitment is an early event during the establishment of endometriotic lesions (Bacci et al. 2009). Using mouse models where syngeneic endometrial tissue was injected in the peritoneal cavity, the authors selectively targeted macrophages by intraperitoneal injections of clodronate liposomes. Whereas the endometrial tissue retained the ability to adhere to the peritoneal wall and to invade the basement membrane in the absence of macrophages, the lesions failed to grow further. Furthermore, depletion of macrophages after the endometriotic lesions had established, prevented the blood vessels from reaching the inner mass of the lesions, which failed to grow (Bacci et al. 2009). In agreement with this study, Haber and colleagues showed that peritoneal-macrophage depletion using liposomal alendronate attenuated the initiation and growth of endometriotic lesions in a rat model of endometriosis (Haber et al. 2009).

Macrophages seem to sustain endometriotic lesion growth by increasing local bioavailability of VEGFA, whose expression in macrophages is regulated by ovarian steroids (McLaren et al. 1996). In addition, peritoneal macrophages may secrete TNF-α and IL-6, which function in a positive feed-forward loop to amplify autocrine VEGFA secretion (Lin et al. 2006b). Finally, macrophages may support angiogenesis in endometriotic lesions by inducing a proangiogenic phenotype in endometriotic stromal cells; indeed, peritoneal-macrophage derived IL-1β was shown to stimulate endometriotic stromal cells to secrete VEGFA and IL-6 (Lebovic et al. 2000). Together, these findings suggest that the recruitment of macrophages is necessary for the successful establishment and growth of endometriotic-lesion associated blood vessels. Besides stimulating angiogenesis, macrophages may stimulate endometrial stromal cells to produce growth factors that support the survival and growth of the endometriotic tissue (Eyster et al. 2010).

Recent reports have provided evidence that macrophages recruited to endometriotic lesions express a “wound-healing” (or M2-like) phenotype that supports lesion vascularization and growth (Bacci et al. 2009; Capobianco et al. 2011). In particular, TEMs were shown to infiltrate human endometriotic lesions and to preferentially localize around angiogenic blood vessels (Capobianco et al. 2011). TEMs were also found to infiltrate endometriotic lesions in a mouse model of the disease. HSV-TK/GCV-based, genetic TEM depletion (De Palma et al. 2003, 2005) inhibited the vascularization and growth of the implanted endometriotic lesions, at least

in part by inducing caspase-3 activation and apoptosis of ECs in newly formed blood vessels (Capobianco et al. 2011). These results suggest that perivascular TEMs may have a role in supporting the viability of ECs recruited to endometriotic lesions (Capobianco et al. 2011). These data support the notion that perivascular macrophages, such as TEMs, provide paracrine (prosurvival, proangiogenic) support to nascent blood vessels during pathological angiogenesis. Furthermore, they corroborate the concept that M2-like activation underlies the proangiogenic functions of macrophages in such angiogenic tissues.

7.3 Macrophage Regulation of Arteriogenesis

In adults, stenosis of main arteries may lead to artery occlusion and ischemic muscle damage. However, increased blood pressure and shear stress in the collateral arteriolar connections may stimulate their enlargement and growth (Fig. 7.2). By this adaptive response, the high blood pressure is released through the collateral arterial network and the function of the occluded artery is partially or completely restored, protecting the tissue from ischemic necrosis (Schirmer et al. 2009). The arteriolar growth following occlusion of a main artery is termed as "arteriogenesis" (Troidl and Schaper 2012).

The concept that macrophages/monocytes play a role in collateral artery growth dates back to 1976, when Scharper and colleagues described the attachment of monocytes to the luminal endothelial surface of collateral arteries and accumulation of macrophages in the perivascular space (Schaper et al. 1976). Macrophages have been shown to adhere to the vascular wall during arteriogenesis, while their activation and secretion of FGF2 and TNF-α play a major role in collateral artery growth (Arras et al. 1998). Further studies showed that blood-monocyte frequency or tissue-macrophage density positively correlate with collateral artery growth (Bergmann et al. 2006; Heil et al. 2002). Indeed, compared to wild-type mice, macrophage-deficient *Csf1*$^{op/op}$ mice show significantly lower macrophage numbers around proliferating arteries and display impaired collateral artery growth and delayed restoration of perfusion following experimental occlusion of the femoral artery (Bergmann et al. 2006).

Several studies analyzed the factors that induce or modulate monocyte/macrophage infiltration at sites of arteriolar growth. Upon the formation of an arteriolar occlusion, the blood flow is directed into pre-existing arteriolar anastomoses, causing enhanced shear stress on the endothelium of the collateral vessels (Heil 2004; Pipp et al. 2004). As a result, ECs release cytokines and growth factors that stimulate SMCs to secrete CCL2 (Heil et al. 2002; Ito et al. 1997; Schirmer et al. 2009). CCL2 then promotes monocyte recruitment and accumulation of proarteriogenic macrophages in peri-arteriolar spaces. Indeed, similar to CSF1-deficient mice, mice either lacking CCL2 or CCR2 display hampered arteriogenesis following arterial occlusion (Heil 2004).

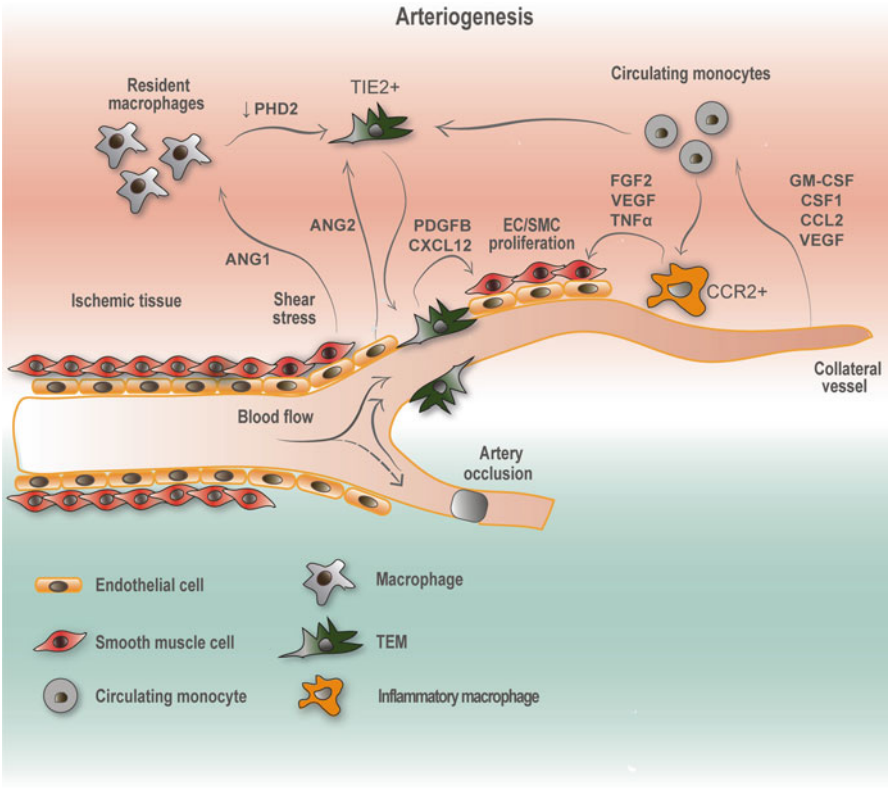

Fig. 7.2 Regulation of arteriogenesis by macrophages. Following occlusion of an artery, the enlargement of the collateral arterial network (arteriogenesis) can alleviate tissue ischemia and rescue blood perfusion. Monocytes/macrophages have been implicated in this process. The upregulation of several monocyte chemoattractants (e.g., VEGFA, CCL2, CSF1, GM-CSF, ANG2, and others) at the site of ischemia promotes monocyte recruitment from the circulation and their differentiation into inflammatory macrophages. These secrete both proangiogenic and proarteriogenic (e.g., VEGFA, FGF2, and TNFα) factors that enhance EC and SMC proliferation. Furthermore, the ischemic upregulation of ANG1 may induce resident macrophages to further differentiate into TEMs, a process that may depend on the downregulation of the oxygen sensor PHD2. TEMs could then enhance EC and SMC survival or proliferation, at least in part, through their secretion of CXCL12 and PDGFB

Monocytes need to adhere to the endothelium of collateral arteries in order to promote arteriogenesis. This process was shown to be mediated by the intercellular adhesion molecule (ICAM)-1, expressed on ECs, and the leukocyte integrin, macrophage-1 antigen (MAC-1), expressed on monocytes. In vivo blockade or genetic knockout of ICAM-1 abolished the stimulatory effect of CCL2 on artery growth (Hoefer et al. 2004). The granulocyte colony-stimulating factor (GM-CSF) has been shown to promote macrophage accumulation and arteriogenesis in a mouse model of femoral artery occlusion (Lee et al. 2005). Furthermore, infusion of

GM-CSF in proximity to the acutely occluded femoral artery of rabbits markedly reduced monocyte apoptosis and increased the arteriogenic response (Buschmann et al. 2001). Of note, GM-CSF did not influence monocyte transmigration or adhesion to cultured ECs (Buschmann et al. 2001), suggesting that it exerts its proarteriogenic functions mainly by enhancing monocyte/macrophage survival.

Macrophages are thought to promote arteriolar growth mainly through paracrine effects on SMCs. Once in the periarteriolar region, macrophages produce growth factors, such as VEGFA and FGF2 (Arras et al. 1998; Ziegelhoeffer et al. 2004), which enhance the motility and proliferation of SMCs, as well as proteases that digest the ECM to provide space for newly recruited SMCs (Hamm et al. 2013; Heil 2004; Pöling et al. 2011; Sato et al. 2000; Schirmer et al. 2009).

Arteriogenesis is associated not only with changes in monocyte/macrophage recruitment (Bergmann et al. 2006; Heil et al. 2002), but also in their activation and functional polarization (Hamm et al. 2013; Takeda et al. 2011; Troidl et al. 2013). Takeda and colleagues used a model of femoral artery occlusion in mice heterozygous for the oxygen sensor, prolyl hydroxylase-2 (PHD2) (Takeda et al. 2011). PHDs are essential negative regulators of HIFs, so genetic *Phd2* haplodeficiency mimics hypoxia (Mazzone et al. 2009; Nangaku et al. 2007). Interestingly, *Phd2* haplodeficient mice (*Phd2*$^{+/-}$) displayed preformed collateral vessels that preserved limb perfusion and alleviated tissue ischemia following artery occlusion. Of note, this effect was mediated, at least in part, by the expansion of "wound-healing" (M2-like) macrophages (Takeda et al. 2011). Furthermore, gene expression profiling studies showed that these macrophages displayed a potentially "proarteriogenic" phenotype, characterized by enhanced expression of CXCL12, PDGFB, and TIE2. *Phd2*$^{+/-}$ macrophages enhanced SMC migration, activation, and growth compared to wild-type macrophages, and in vitro knockdown of both *Cxcl12* and *Pdgfb*, which are known to stimulate SMC recruitment and proliferation, abolished the enhanced response of SMCs to *Phd2*$^{+/-}$ macrophages. These data therefore suggest a mechanism whereby macrophages promote arteriogenesis (Takeda et al. 2011).

ANG/TIE2 signaling has been identified as a major modulator of arteriogenesis in the ischemic muscle. Overexpression of ANG2 increased recovery of perfusion in the ischemic mouse hindlimb, while reperfusion was markedly reduced in *Tie2-deficient* mice (Lekas et al. 2012). Furthermore, a chemical inhibitor of ANG2 impaired arteriogenesis in a mouse model of hindlimb ischemia, a phenotype that was associated with the decreased recruitment of CD11b+ myeloid cells to the ischemic muscle (Tressel et al. 2008). Among myeloid cells, TEMs were recently reported to enhance both angiogenesis (Patel et al. 2013) and arteriogenesis (Hamm et al. 2013) in limb ischemia models. Hamm and colleagues have shown that increased expression of ANG1 in the ischemic muscle promotes the in situ differentiation of macrophages into M2-like macrophages characterized by enhanced TIE2 expression (Hamm et al. 2013). Upregulation of TIE2 in macrophages relied on ANG1-mediated PHD2 repression. Indeed, ANG blockade by a soluble TIE2 trap (sTIE2) prevented both the downregulation of PHD2 in the macrophages and their phenotypic switch. Of note, either the conditional silencing of *Tie2* in TEMs or their genetic depletion (using a HSV-TK/GCV-based suicide-gene strategy) increased

ischemic necrosis following artery occlusion (Hamm et al. 2013). Along these lines, Patel and colleagues reported a clinically relevant role for TEMs in patients with critical limb ischemia (CLI). These authors found that CLI patients had increased levels of circulating and muscle-resident TEMs compared to healthy subjects or non-ischemic muscle of CLI patients, a phenotype that could be reverted upon removal of ischemia (Patel et al. 2013). Interestingly, TEMs isolated from the circulation of patients with CLI displayed greater proangiogenic activity than TIE2-negative monocytes in in vitro angiogenesis assays, suggesting that ischemia enhances the proangiogenic functions of human TEMs (Patel et al. 2013). Together with the study of Hamm and colleagues (Hamm et al. 2013), these findings corroborate the notion that macrophage expression of TIE2 plays an important role in modulating the proangiogenic and proarteriogenic functions of M2-like macrophages (Emanueli and Kränkel 2013).

7.4 Macrophage Regulation of Lymphangiogenesis

The main physiological functions of the lymphatic system are to regulate tissue-fluid homeostasis, absorption of dietary fats, and immune cell trafficking. Lymphatics serve as a conduit for soluble antigens and antigen-presenting cells (e.g., DCs and macrophages) from the peripheral tissues to the lymph-nodes and secondary lymphoid organs, where antigens are presented to B and T-cells to initiate immune responses (Cao 2005; Tammela and Alitalo 2010). Lymphatics are present in all vascularized tissues except the BM and central nervous system.

Lymphangiogenesis, the formation of new lymphatic vessels, occurs in adult tissues during inflammation, wound healing and tumor development. In cancer, invasive cancer cells use the blood and the lymphatic vessels to spread and metastasize to lymph-nodes and distant organs. Cancer cells that have intravasated into the lymphatic system (generally via peri-tumporal lymphatics) can reach the venous circulation and thus spread to distant sites. The role of tumor lymphatics in metastatic cancer spread is well established and has been described elsewhere (Cao 2005; Ito et al. 2011; Kodera et al. 2011; Larrieu-Lahargue et al. 2012; Skobe et al. 2001; Stacker et al. 2002).

Whereas during embryonic development the first lymphatic vessels sprout from the cardinal vein, in adults lymphangiogenesis occurs primarily via sprouting of pre-existing lymphatics (Cao 2005; Tammela and Alitalo 2010). Prospero-related homeodomain transcription factor (PROX1) and VEGFR3 are essential for the differentiation of blood vessel ECs to lymphatic ECs (LECs) and sprouting, respectively (Cao 2005; Stacker et al. 2002). VEGFC and D are the main inducers of lymphangiogenesis in development, inflamed, and tumoral tissues (Baluk et al. 2009; Cao 2005; Karkkainen et al. 2004; Mandriota 2001; Rissanen et al. 2003; Skobe et al. 2001; Tammela and Alitalo 2010). Both VEGFC and D bind and activate VEGFR3, whose expression is restricted to LECs and sprouting ECs (Tammela and Alitalo 2010). FGF2 may also promote lymphangiogenesis cooperatively with VEGFC in

the mouse retina and tumor models (Cao et al. 2012). Of note, multiple cell types may secrete VEGFC in response to proinflammatory cytokines such as TNF-a, including macrophages and granulocytes (Adams and Alitalo 2007; Baluk et al. 2009).

The transcription factor NF-kB plays an important role in lymphangiogenesis, as it transcriptionally regulates the expression of VEGFR3 (Flister et al. 2010). Furthermore, NF-kB is constitutively activated in LECs and induces the production of leukocyte chemoattractants such as CCL2 and CCL5, resulting in leukocyte homing to the lymphatic vessels and draining lymph-nodes (Kang et al. 2009). Myeloid cells are often detected in areas of new lymphatic-vessel growth, and it has been suggested that these cells contribute to lymphangiogenesis by providing paracrine factors such as VEGFC and D (Ito et al. 2011; Nucera et al. 2011; Ran and Montgomery 2012; Zumsteg et al. 2009). Among leukocytes, macrophages are an important source of lymphangiogenic growth factors. Accordingly, *Csf1*$^{op/op}$ macrophage-deficient mice display delayed postnatal lymphatic-vessel development, whereas pharmacological inhibition of the CSF1/CSF1R axis between postnatal days 8 and 15 results in reduced lymphatic vessel branching. Macrophages were also shown to modulate lymphatic vessel density in postnatal tissues (Kubota et al. 2009), secrete VEGFC and D and promote lymphangiogenesis in a model of suture-induced, inflammatory corneal neovascularization in adult mice (Cursiefen et al. 2004). Macrophage-derived VEGFC was shown to promote inflammation-associated lymphangiogenesis in other pathophysiological conditions, such as skin inflammation (Kataru et al. 2009) and wound healing (Maruyama et al. 2007). TAMs may be a relevant source of both VEGFC and D also in some tumor types, such as cervical cancers (Schoppmann et al. 2002). Importantly, breast cancer patients with lymphnode metastasis show a positive correlation between numbers of VEGFC+ macrophages and lymphatic vessel density (Ding et al. 2012; Schoppmann et al. 2006).

Bohmer and colleagues described a population of embryonic monocytes/macrophages identified by their expression of the Syk tyrosine kinase and high levels of proangiogenic (FGF2, PDGFB, MMP2, and MMP9) and lymphangiogenic growth factors, namely VEGFC and D (Böhmer et al. 2010; Nucera et al. 2011). Interestingly, Syk-deficient embryos displayed lymphatic hyperplasia in the developing skin caused by accumulation of these lymphangiogenic monocytes. Intriguingly, the Syk-expressing monocytes were found to also express TIE2 (Böhmer et al. 2010) and to resemble the proangiogenic TIE2+ macrophages found in tumors and embryos (Nucera et al. 2011; Pucci et al. 2009).

Gordon and colleagues identified a subset of mouse embryonic, LYVE1+ dermal macrophages that were intimately associated with the developing lymphatic vasculature (Gordon et al. 2010). Interestingly, the gene expression profile of these LYVE1+ macrophages was reminiscent of that of embryonic and tumor-derived TEMs (Pucci et al. 2009). Although these embryonic dermal macrophages promoted the proliferation of primary embryonic dermal LECs in ex vivo co-culture experiments, embryos lacking such dermal macrophages (i.e., *PU.1*$^{-/-}$ and *CSF1R*$^{-/-}$ mice) did not have impaired lymphatic development but displayed hyperplastic lymphatic vessels in the skin (Gordon et al. 2010). The authors found that the

LYVE1+ dermal macrophages only provided 10 % of the prolymphangiogenic molecules present in the dermal microenvironment (Gordon et al. 2010). These findings suggest that while skin macrophages may be dispensable for VEGFC/D-dependent LEC proliferation, they likely modulate lymphatic vessel morphogenesis by limiting excessive LEC growth, as also shown in the context of retinal angiogenesis (Harvey and Gordon 2012; Gordon et al. 2010; Nucera et al. 2011).

In addition to their production of classic prolymphangiogenic or ECM-remodeling growth factors (Karkkainen et al. 2004; Kataru et al. 2009; Maruyama et al. 2007; Schoppmann et al. 2002), macrophages may regulate lymphangiogenesis through additional mechanisms. For example, it was proposed that monocytes/macrophages may transdifferentiate into LECs and incorporate into lymphatics during pathological lymphangiogenesis (Kerjaschki et al. 2006; Maruyama et al. 2005; Religa 2005). However, Gordon and colleagues showed that LECs arise independently of the myeloid lineage during both embryogenesis and tumor lymphangiogenesis (Gordon et al. 2010), in agreement with previous studies in mouse models of cancer (He et al. 2004). Irrespective of their ability to directly transdifferentiate into LECs, there is now compelling evidence for monocytes/macrophages to modulate the growth of lymphatic vessels both in development and pathological conditions (Harvey and Gordon 2012; Nucera et al. 2011).

7.5 Concluding Remarks

As discussed above, the proangiogenic activity of macrophages (or subsets thereof) may involve the secretion of proangiogenic growth factors as well as the production of proteolytic enzymes that can both facilitate vessel sprouting in the ECM and activate latent growth factors embedded therein. Furthermore, recent data indicate that the physical association of macrophages with sprouting blood vessels may be essential in later stages of vascular development, e.g., during the process of vascular anastomosis that occurs downstream to VEGFA-induced vessel sprouting. Such contact-dependent macrophage–EC interactions may be bidirectional, as in vitro co-culture studies showed that EC monolayers can support the differentiation of proangiogenic macrophages directly from myeloid progenitors (He et al. 2012). These intriguing observations suggest that dynamic angiogenic vascular niches might also exist in vivo, for example, in tumors where sprouting blood vessels and immature myeloid cells engage in reciprocal interactions (Baer et al. 2013). Although the ANG2/TIE2 (Mazzieri et al. 2011), CXCL12/CXCR4 (Grunewald et al. 2006), CSF1/CSF1R (He et al. 2012), and DLL4/NOTCH (Outtz et al. 2011) signaling pathways may all be involved, the molecular mechanisms that regulate contact-dependent, macrophage–EC interactions are still poorly understood (Baer et al. 2013).

While generally regarded as proangiogenic (Baer et al. 2013; Nucera et al. 2011; Squadrito and De Palma 2011; Sunderkötter et al. 1994), macrophages can also exert context-dependent antiangiogenic functions. For example, fetal macrophages have been shown to both promote and limit the formation of vascular intersections in developing neural tissues (Baer et al. 2013; Fantin et al. 2010; Lobov et al. 2005;

Rao et al. 2007; Rymo et al. 2011; Stefater et al. 2011). Whereas this may reflect anatomical-site or developmental-stage specific macrophage functions, possibly modulated by ECs or other stromal cells, several questions currently remain unanswered (Baer et al. 2013). Do macrophages that facilitate vascular growth (including angiogenesis and vessel anastomosis) differ from the macrophages that promote vascular regression? What are the microenvironmental signals that modulate opposite functions by ostensibly similar cells? Do signals released by differently activated ECs instruct such divergent macrophage functions? The identification of the molecular signals, both extrinsic and intrinsic, that regulate pro- versus antiangiogenic activity of macrophages may provide clues to reprogram these cells toward an angiostatic/antiangiogenic function in disease conditions characterized by excessive angiogenesis, such as tumors.

High macrophage numbers often correlate with high vascular density in both mouse and human cancer, indicating that macrophages are an important source of proangiogenic factors in tumors (Squadrito and De Palma 2011). In several mouse models of cancer (e.g., MMTV-PyMT), TAMs appear to contribute to the plethoric and highly chaotic tumor-associated vasculature by delivering "excessive" proangiogenic stimuli. Indeed, tumor blood vessels are converted into a "normalized" form upon the partial removal of TAMs or interference with their proangiogenic programs (DeNardo et al. 2011; Mazzieri et al. 2011; De Palma et al. 2008; Rolny et al. 2011; Stockmann et al. 2008). Several recent reports have shown that antagonizing the proangiogenic functions of TAMs, or depleting them from tumors, indeed enhances the delivery and efficacy of chemotherapy, possibly as a consequence of "vascular normalization" and better tumor perfusion (DeNardo et al. 2011; De Palma and Lewis 2013; Rolny et al. 2011; Squadrito and De Palma 2011; Stockmann et al. 2008). It should be noted, however, that whereas substantial data indicate that rapidly growing mouse tumors are dependent on TAMs (or subsets thereof, such as TEMs) to mount a proficient angiogenic response (De Palma et al. 2003; Squadrito and De Palma 2011), it is currently unknown whether slowly growing tumors (like early-stage human malignancies) would also rely on the contribution of these cells to build up new blood vessels. Furthermore, myeloid cells such as neutrophils, immature myeloid cells, and mast cells may also contribute to support tumor angiogenesis (Shojaei and Ferrara 2008). Remarkably, neutrophils and immature myeloid cells may rescue tumor angiogenesis in TAM-depleted tumors, at least in some mouse models of cancer (Pahler et al. 2008).

TAMs not only express proangiogenic programs that can enhance pathological tissue vascularization, but may also limit the efficacy of antiangiogenic therapy (Bergers and Hanahan 2008; De Palma and Lewis 2013; Shojaei and Ferrara 2008; Shojaei et al. 2007; Ahn et al. 2010; Kozin et al. 2010; Kioi et al. 2010). The selective destruction of tumor blood vessels by high-dose antiangiogenic drugs or vascular-disrupting agents (VDAs) enhances tumor hypoxia and necrosis, which increase tumor levels of several hypoxia-regulated monocyte/TAM chemoattractants. In turn, the de novo recruitment of myeloid cells, including macrophages, may accelerate tumor revascularization (Bergers and Hanahan 2008; Du et al. 2008; De Palma and Lewis 2013). For example, when mouse mammary tumors were treated with the VDA combretastatin-A4-phosphate (CA-4-P), vessel collapse and

increased tumor hypoxia were associated with elevated tumor levels of CXCL12 and increased tumor infiltration by TEMs (Welford et al. 2011). Blocking this CA-4-P-induced recruitment of TEMs to the tumors, either using a CXCR4 antagonist or by genetic TEM depletion (De Palma et al. 2003, 2005), markedly increased the efficacy of CA-4-P treatment (Welford et al. 2011). These observations suggest that enhanced TEM/TAM recruitment may protect tumors from the effects of vascular-targeted drugs, and possibly contribute to drive evasive resistance to the treatment (Bergers and Hanahan 2008; De Palma and Lewis 2013). It remains to be seen whether tumor resistance (or refractoriness) to angiogenesis inhibitors is associated with the enhanced recruitment (or a more pronounced proangiogenic phenotype) of TAMs in cancer patients.

Duly polarized, proangiogenic macrophages could also be employed in the setting of cell therapy to promote the therapeutic revascularization of ischemic tissues. Indeed, cell-based therapies may represent a valuable strategy to achieve sustained production of the complex mixture of growth factors that is required for the effective revascularization of either acutely or chronically ischemic tissues. However, the use of unselected BM or peripheral blood-derived cells has so far provided equivocal clinical results (Fadini et al. 2010; Losordo and Dimmeler 2004). On the other hand, recent preclinical data have suggested that the use of selected proangiogenic/proarteriogenic macrophages like TEMs may help enhance tissue revascularization following experimental limb ischemia (Patel et al. 2013). It will, therefore, be of great importance to identify cell culture conditions that can induce and expand *bona fide* proangiogenic macrophages from unselected monocytes or other cell sources. In this regard, the generation of TEM-like, proangiogenic monocytes/macrophages from human embryonic stem cells (ESCs) has been reported in one study (Klimchenko et al. 2010). These ESC-derived, $TIE2^{+}$ monocyte-like cells expressed high levels of genes encoding matrix-remodeling enzymes, proangiogenic factors, and scavenger receptors, and promoted vascular remodeling in human tumors grown in mice (Klimchenko et al. 2010). The generation of TEM-like cells, ideally from autologous monocytes or BM-derived hematopoietic progenitors, may provide a valuable source of proangiogenic cells for the treatment of ischemia, injured tissues, or chronic wounds.

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Part III
Macrophages in Different Tissues

Chapter 8
Airway Macrophages: Sentinels of Health

Tracy Hussell, Thomas J. Bell, and Toshifumi Fujimori

The lower airspaces are lined by a single layer of epithelium in close contact with a vast blood supply to facilitate gaseous exchange. This delicate surface is also exposed to a myriad of environmental factors, some of which may be pathogenic. Inflammation in the lung impedes its primary function and for this reason a specialised environment exists that prevents innate immune cell activation unless the threat is significant. Airway macrophage are an intriguing cell type that dominate in health and appear to be very different from macrophage anywhere else in the body. They occupy a unique niche, dominated by airway epithelium and exposed to innocuous environmental antigens and allergens that have avoided impaction in the upper nasopharynx. Significant advances have been made in the last few years in how an airway macrophage discriminates between inhaled harmless environmental particles (that often contain proteolytic activity, mimics of TLR signalling complex molecules and lipid-binding activity, and the ability to engage pattern recognition receptors (Wills-Karp et al. 2010)) and more pathogenic microorganisms. They express a unique range of surface receptors and are not as readily activated compared to macrophage elsewhere in the body. The factors causing the altered phenotype and function is an area of intense investigation since these pathways may be altered in acute and chronic lung inflammation and provide clues to disease pathogenesis. In recent years evidence has emerged that the respiratory epithelium plays a significant role in controlling the activity of innate immunity; in particular airway macrophage.

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8.1 Airway Macrophage in Health

There are three major macrophage populations in the respiratory system; bronchial macrophage, alveolar macrophage, and interstitial macrophage. Bronchial macrophage are normally isolated from induced sputum samples, while alveolar macrophage are found in the alveolar lumen and are normally recovered from broncho-alveolar lavage fluid. Interstitial macrophage exist in the lung parenchyma where they interact with interstitial lymphocytes (Balhara and Gounni 2012). Alveolar macrophage are the most common antigen-presenting cells in the respiratory system and constitute about 95 % of all cells recovered from broncho-alveolar lavage at homeostasis (Gordon and Read 2002). The alveolar macrophage population in the lungs turn over slowly, with an estimated half-life of 30–60 days and turnover rate of approximately 40 % over a period of 1 year in the healthy lung. Although alveolar macrophage can be derived from blood monocytes, a significant proportion of alveolar macrophage are thought to be generated from a self-renewing population of progenitors in the lung parenchyma (Guth et al. 2009).

Compared to macrophage populations in other sites of the body, alveolar macrophage are considerably larger, and phenotypically more closely resemble the phenotype of dendritic cells (Laskin et al. 2001). The markers described here were identified on mouse macrophages, but will also be relevant to human macrophage populations. Dendritic cells are classified by high CD11c expression, high level of MHC class II expression, and expression of co-stimulatory markers such as CD40, CD80, and CD86. Macrophage, on the other hand, are typically classified by high expression levels of CD11b and MHC class II. Alveolar macrophage express low levels of CD11b and express markers such as CD11c that are typically used to characterise dendritic cells (Guth et al. 2009).

Unlike other macrophage populations, alveolar macrophage express high levels of CD11c, which is normally limited to dendritic cells and some subsets of natural killer cells and T-cells (van Rijt et al. 2005). CD11c is a member of the leukointegrin family which binds to a wide range of ligands. One of the proposed properties of CD11c is its capability to mediate the complement-dependent phagocytosis of iC3b opsonised elements, and in fact CD11c is also known as complement receptor 4 (Malhotra et al. 1986). It was later shown, however, that CD11c is capable of mediating phagocytosis without the presence of iC3b, and may be able to recognise the bacterial cell wall component LPS directly (Sadhu et al. 2007). CD11c may also be involved in antigen presentation in dendritic cells as implicated by an inability of Langerhans cells to induce allogeneic proliferation of T-cells when CD11c is blocked (Schwarting et al. 1985). Furthermore, CD11c has been shown to interact with various cell adhesion molecules involved in the recruitment of antigen-presenting cells to the site of inflammation such as ICAM-1, ICAM-2, and ICAM-4 (Sadhu et al. 2007). Alveolar macrophage also express high levels of DEC-205, not normally expressed by macrophage populations from other parts of the body and only expressed by a subset of dendritic cells (Staunton et al. 2006).

Although alveolar macrophage and pulmonary dendritic cells are phenotypically similar in many ways, their responses to inflammation are characteristically distinct.

Recruitment of alveolar macrophage and pulmonary dendritic cells occurs with different kinetics following infection with the bacteria *Streptococcus pneumoniae*, with only an increase in the number of alveolar macrophage observed (Kirby et al. 2006). Expression of CD11b on alveolar macrophage is associated with maturation and becomes significantly up-regulated following treatment with granulocyte/macrophage colony-stimulating factor (GM-CSF). Conversely, pulmonary dendritic cells constitutively express CD11b regardless of their resting/activated status. Antibody blockade of CD11b has demonstrated the critical role for CD11b in the recruitment of alveolar macrophage, but not pulmonary dendritic cells, into the respiratory system following bacterial challenge (Kirby et al. 2006).

There is evidence that there are distinct populations of airway macrophage. Monocytes which are recruited into the alveolar space show largely the same phenotype as blood monocytes; typically positive for the surface markers F4/80, CD11a, CD11b, CD18, CD49d, and CD62L. In contrast, CD14 and TNF-α expression is up-regulated by monocytes that are recruited into the alveolar space. These markers clearly differentiate between the recruited monocyte population from the peripheral blood and the resident alveolar macrophage as monocytes recruited into the alveolar space during inflammation retain the phenotypic characteristics of peripheral blood monocytes, but significantly up-regulate CD14 expression and are more pro-inflammatory when compared to resident alveolar macrophage (Maus et al. 2001). See Table 8.1 for a comparison of the surface marker expression of various macrophage populations.

Table 8.1 A comparison of surface marker and negative regulator expression by mouse macrophage from a number of different sites around the body and dendritic cells

Lineage markers	Peritoneal macrophage	Peripheral blood monocytes	Recruited monocytes	Alveolar macrophage	Dendritic cells
Axl	Low	Low	Intermediate	High	
CD11a	High	High	High	Low	
CD11b	High	High	High	High	High
CD11c	Low	Low	Intermediate	High	High
CD14	High	Low	High	Low	High
CD18	High	High	High	Low	Low
CD49d		High	High		
CD62L		High	High		
CD68	High	High	High	High	Low
CD163	High	High	High	Low	Some high
CD200R	Low	Low	Intermediate	High	
DEC-205	Low	Low		High	Some high
F4/80	High	Low	High	High	Low
MerTK	Intermediate	Low	Intermediate	High	
TREM-1	Low	Low		Intermediate	
TREM-2	Low	Low		Intermediate	

Data combined from Hussell unpublished observations, and Brauner et al. (1998), Fock et al. (2007), Lau et al. (2004), Lundahl et al. (1996), Maus et al. (2001), Sadhu et al. (2007), Snelgrove et al. (2008), and Staunton et al. (2006)

The unique features of alveolar macrophage may reflect the unusual micro environment in which they reside. The maturation and survival of alveolar macrophage depends (at least in part) on locally secreted cytokines such as macrophage colony-stimulating factor (M-CSF). The respiratory epithelium is also a rich source of GM-CSF, and therefore alveolar macrophage are immersed in a high concentration of GM-CSF at all times. It has been shown in vivo that high levels of GM-CSF in the alveolar space are necessary for generation of the CD11c high alveolar macrophage phenotype (Sadhu et al. 2007). Furthermore, alveolar macrophage are exposed to high oxygen tension and high levels of surfactant proteins (SP), SP-A and SP-D in particular, which are known to have immunomodulatory effects (Guth et al. 2009). All of these factors together promote the development of alveolar macrophage with unusual characteristics and a distinctive dendritic cell like phenotype.

Airway macrophage are the key sentinels of the airway and at homeostasis actively suppress both adaptive and humoral immunity via the production of nitric oxide, prostaglandins, interleukin-4 and -10 (IL-4, IL-10), and transforming growth factor-β (TGF-β) (Lipscomb et al. 1993; Thepen et al. 1994). At homeostasis airway macrophage are in close proximity and in some cases even adhere to airway epithelial cells, and are subject to regulation by epithelium-secreted factors. Surfactant proteins A and D (SP-A and SP-D) which are abundant in the epithelial lining fluid and produced in the lower airways by alveolar type II cells (Boggaram 2003) reduce the phagocytic activity of alveolar macrophage by binding to a macrophage-expressed negative regulator, signal inhibitory regulatory protein (SIRP)-α (Janssen et al. 2008). This regulatory role in normal conditions is exemplified in SP-D-deficient mice that show constitutive alveolar macrophage activation (Yoshida and Whitsett 2006). SP-A binds (via its carbohydrate recognition domain) to Toll-like receptor (TLR)-4, TLR-2, MD-2, and CD14 (Ariki et al. 2012; Yamada et al. 2006), thereby impeding interaction between airway macrophage and their respective TLR ligands.

8.2 Negative Regulators of Airway Macrophage

CD200R is expressed at high levels on airway macrophage and transmits a negative regulatory signal. CD200R binds to CD200 which is expressed on thymocytes, B cells, activated T-cells, neurons, follicular dendritic cells, endothelium, epithelium, and in reproductive organs. Limited studies exist characterising the downstream signalling events following CD200R ligation, however in mast cells phosphorylation of the cytoplasmic tail of CD200R is induced upon ligation with CD200 and leads to the recruitment of adaptor proteins Dok-1 and Dok-2, which are in turn phosphorylated and associate with RasGAP and SH2-containing inositol phosphatase (SHIP), ultimately inhibiting the phosphorylation of ERKs, p38, and JNK. The intracellular tail of CD200 is 67 amino acids shorter than that of its ligand, and lacks phosphorylation sites, suggesting that the action of CD200 ligation is solely delivered through the receptor and is not bi-directional.

In the lung, CD200R is expressed at high levels on airway macrophage compared to macrophage from more sterile sites including those within the lung parenchyma and spleen, suggesting the importance of this interaction in maintaining a high threshold of immune activation at mucosal sites. Its ligand, CD200, is expressed on the luminal aspect of the lower airway epithelial cells in mice. Airway macrophage from CD200R deficient mice produce significantly higher levels of pro-inflammatory cytokines such as TNF-α in response to interferon (IFN) than wild-type controls, and also spontaneously activate in the absence of IFN. CD200 deficient mice develop excessive inflammation following influenza infection, whilst artificially enhancing the CD200–CD200R negative signal using CD200 antibody during influenza infection reduces inflammation, and disease severity without hindering viral clearance. Interestingly alveolar macrophage present in resolution from influenza infection express higher levels of CD200R than those at homeostasis, and this heightened regulation in resolution is in part responsible for susceptibility to secondary bacterial infections, since CD200R deficient mice are protected from bacterial complications (Goulding et al. 2011; Snelgrove et al. 2008).

TREM-2 (Triggering Receptors Expressed on Myeloid Cells) which is found on many myeloid cells, including macrophage, microglia, osteoclasts, and dendritic cells is structurally similar to TREM-1. It consists of three domains: an extracellular V-type immunoglobulin-like domain, a transmembrane region containing lysine residues with positive charge, and a short cytoplasmic tail (Colonna 2003; Hamerman et al. 2005). These related receptors both signal through the adaptor protein DAP12. However, whilst TREM-1 has pro-inflammatory effects, multiple studies suggest that TREM-2 dampens TLR signalling and has an anti-inflammatory role. We observe high levels of TREM-2 on airway macrophage and, like CD200R, expression increases in resolution of inflammation (Hussell unpublished observations). After stimulation with an agonistic antibody, the transmembrane domain of TREM-2 phosphorylates the ITAM on DAP12, which recruits the tyrosine kinase SYK and leads to the activation of PI3K and MAPK (Bouchon et al. 2001; Jiang et al. 2002). Other downstream events include ERK phosphorylation and Ca^{2+} mobilisation. Unlike TREM-1, TREM-2 ligation does not induce the degradation of IκB-α and the subsequent nuclear translocation of NF-κB (Bouchon et al. 2001). Initial work suggests that TREM-2 suppresses macrophage TNF-α and IL-6 production and promotes an anti-inflammatory state in microglia (Hamerman et al. 2005). Gene silencing of TREM-2 results in increased production of TNF-α in response to the TLR-2/6 ligand zymosan and the TLR-9 ligand CpG (Hamerman et al. 2006). Consistent with this finding, bone marrow derived macrophage from TREM-2 deficient mice secrete significantly more TNF-α and IL-6 in response to LPS than wild-type controls (Turnbull et al. 2006). Additionally TREM-2 signalling inhibits cell motility by reducing β2 integrin expression via plexin A1 and semaphorin receptors (Ford and McVicar 2009).

TAM receptors (*T*yro3, *A*xl and *M*erTK) also play a significant role in lung inflammation and Axl and MerTK are differentially expressed on airway macrophage with different kinetics during an inflammatory response. These are tyrosine kinase receptors and are expressed on a broad range of myeloid cells including

macrophage. TAM receptors recognise apoptotic cells indirectly via ligation of GAS6 or Protein S; which are structurally closely related and act as bridging molecules between phagocytic cells and externalised phosphatidylserine on apoptotic cells (Stitt et al. 1995). Ligation ultimately leads to efferocytosis of the apoptotic cell, which is invariably an anti-inflammatory process. TAM receptor signalling contributes to the anti-inflammatory effect of efferocytosis by initiating a negative feedback loop which dampens TLR signalling. On up-regulation, TAM receptors physically associate with the type 1 interferon receptor (IFNAR) thereby interfering with the downstream IFNAR-STAT-1 signal transduction cascade. This leads to a reduction in NF-κB levels and the subsequent broad de-sensitisation of TLRs by induction of Suppressor of Cytokine Signalling-1 and -3 (SOCS1 and 3) (Rothlin et al. 2007).

How these three receptor families interact to limit airway macrophage activation is shown in Fig. 8.1.

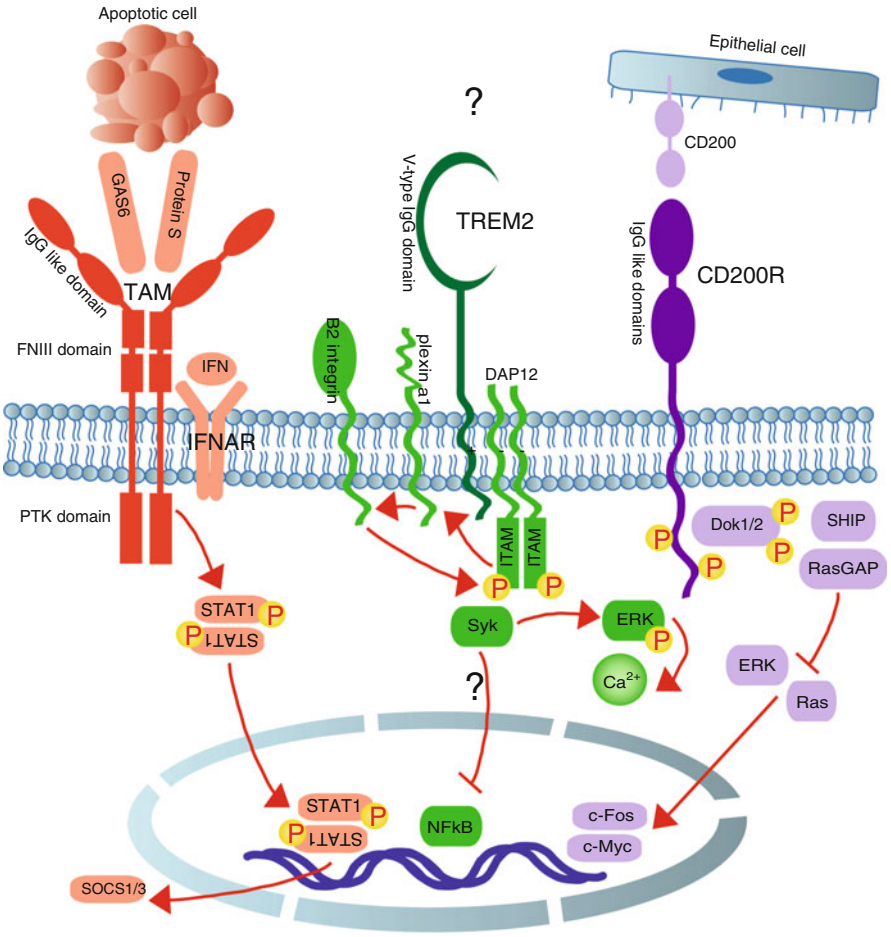

Fig. 8.1 Current understanding of TAM, TREM-2, and CD200R signalling pathways. TAM receptors (*red* and signalling pathways *pink*) are tyrosine kinases composed of two extracellular

8.3 Epithelial Regulatory Factors that Influence Airway Macrophage

Epithelial cells express and secrete a battery of immune modulators whose role is to limit innate inflammation to prevent activation by innocuous antigens. On the epithelium CD200 transmits a regulatory signal through the CD200 receptor which is expressed at high levels on airway macrophage (Snelgrove et al. 2008). Additionally, the integrin αvβ6 binds to and activates latent TGFβ (Munger et al. 1999; Takabayshi et al. 2006), the IL-33 receptor ST2 sequesters MyD88 and MAL (Liew et al. 2010), surfactant proteins bind to pathogen-associated molecular patterns (PAMPs) and subsequently via their C-terminal to signal inhibitory regulatory protein (SIRPα, CD172a) (Gardai et al. 2003), and MUC1, a transmembrane mucin-like glycoprotein suppresses alveolar macrophage responses to TLRs 2–5, 7 and 9 (Ueno et al. 2008). IL-10 and TGFβ also promote further expression of CD200R on alveolar macrophage (Snelgrove et al. 2008). Induction of inflammation therefore requires antigen, but also a reduction in these regulatory pathways that occurs when structural/epithelial integrity is lost (Fig. 8.2).

In this environment alveolar macrophage are less efficient as antigen-presenting cells, with lower levels of MHC Class II and co-stimulatory molecules compared to tissue-resident counterparts (Steinmuller et al. 2000). Furthermore they are poorly phagocytic (Janssen et al. 2008) and reduce T cell responses via secretion of prostaglandins and TGFβ (Roth and Golub 1993), resulting in T cell inactivation (Balbo et al. 2001; Chelen et al. 1995). Alveolar macrophage are also reported to actively inhibit the antigen presentation function of airway inter-digitating dendritic cells (Holt et al. 1993) that in turn secrete IL-10 (Akbari et al. 2001). The respiratory epithelium therefore effectively sets up a tissue-specific microenvironment (Mayer and Dalpke 2007) that governs the reactivity of airway innate immunity.

Fig. 8.1 (continued) Current IgG like domains, a FNIII domain, and an intracellular PTK domain that indirectly binds to apoptotic cells via Gas6 or protein S. Co-operation with the interferon alpha receptor (IFNAR) leads to phosphorylation of STAT1, which translocates to the nucleus where it upregulates the expression of negative regulators of TLR signalling including SOCS1 and 3. TREM-2 is shown in *dark green* and associated signalling in *light green* is composed of an extracellular V-type IgG domain, a positively charged transmembrane domain and a short cytoplasmic tail. Activation of this receptor leads to recruitment and phosphorylation of the ITAM domain of the adapter protein DAP12. Via recruitment the kinase Syk this causes phosphorylation of ERK and subsequent alteration in Ca^{2+} signalling as well as inhibiting NFκB translocation to the nucleus by as yet unidentified pathways. CD200R (in *purple* with signalling pathways in *light purple*) is composed of two extracellular Ig-like domains and a long cytoplasmic tail containing sites for phosphorylation on ligation of CD200. This leads to the recruitment of Dok1 and 2, the recruitment of SHIP and RasGAP, and the subsequent regulation of ERK and Ras signalling pathways. Ultimately this alters the expression of genes controlled by the transcription factors c-Fos and c-Myc

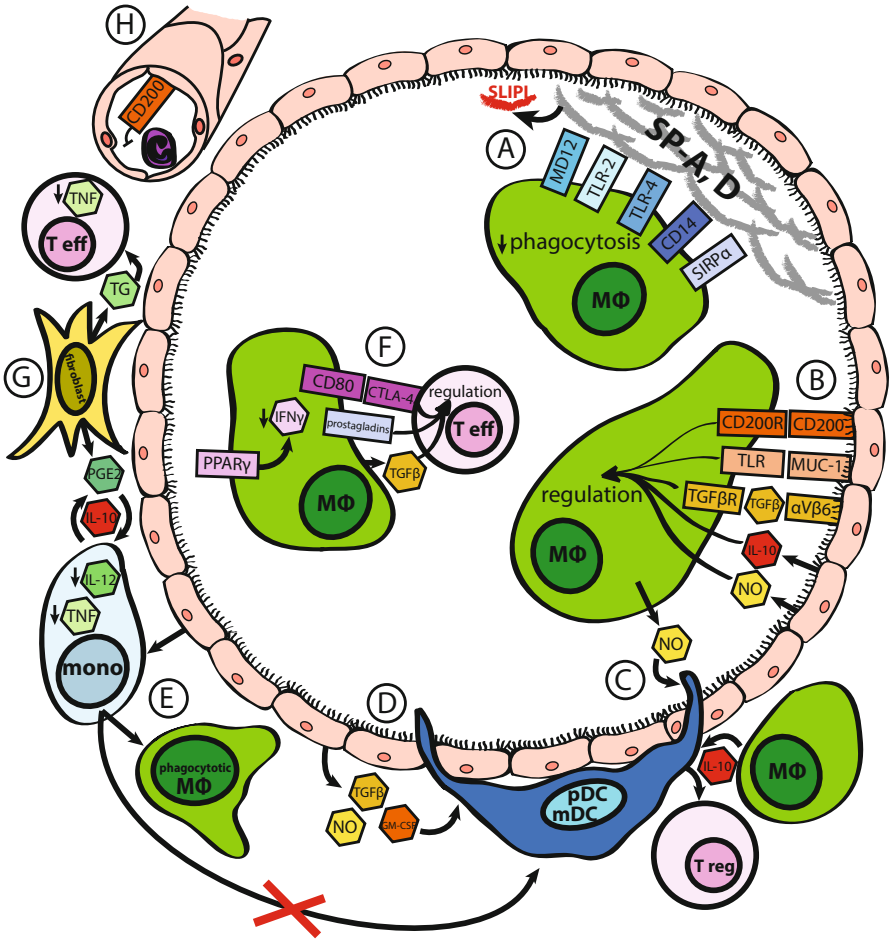

Fig. 8.2 Active maintenance of immunological homeostasis within the lower airways of the respiratory tract. (**a**) Type II epithelial cell derived surfactant proteins suppress alveolar macrophage activation through impeding recognition of TLR ligands and imparting inhibitory signals through negative immune regulator SIRP-α. (**b**) Epithelial cells further limit alveolar macrophage activation through the release of soluble anti-inflammatory mediators TGFβ, nitric oxide (NO) and IL-10, and via cell-contact mediated inhibition imparted by negative regulator CD200 and surface glycoprotein MUC1. (**c**) Plasmacytoid and myeloid DCs promote immunological tolerance to antigen through the release of IL-10 and induction of regulatory T-cells. Activation and maturation of DCs is further suppressed by alveolar macrophage-derived NO and interstitial macrophage IL-10. (**d**) Airway epithelial cells also limit DC responsiveness through TGFβ, NO, and GM-CSF. (**e**) Airway epithelial cells further act to limit the development of an adaptive immune response by preferentially promoting monocyte development into phagocytic macrophage as opposed to more capable antigen-presenting DCs. (**f**) Alveolar macrophage are poor APCs that are incapable of eliciting adaptive immunity owing to low co-stimulatory molecule expression or elevated expression of CD80 that preferentially binds negative T cell regulator CTLA-4. Alveolar macrophage also directly inhibit T cell responses via the secretion of prostaglandins and TGFβ that render T-cells inactive. Alveolar macrophage also constitutively express PPAR-γ that negatively regulates the expression of pro-inflammatory genes. (**g**) Lung fibroblasts act as a reservoir for triglycerides that subsequently protect the lung from oxygen-radical-imposed injury. Fibroblasts further act to suppress inflammation through PGE2-mediated inhibition of monocyte TNF-α and IL-12 production and repression of T cell TNF-α production, activation, and proliferation. (**h**) Endothelia express high levels of CD200 that imparts an inhibitory signal to immune cells as they egress and migrate into the lung. Reprinted from Snelgrove et al. (2011), Copyright 2011, with permission from Elsevier

8.4 Changes in the Epithelium in Acute and Chronic Disease Leading to Aberrant Macrophage Activity

Since macrophage are regulated by the lung microenvironment, it is reasonable to assume that their phenotype and function will change in patients with chronic inflammatory disease such as Chronic Obstructive Pulmonary Disease, Asthma, Idiopathic Pulmonary Fibrosis, or Cystic Fibrosis, where remodelling and repair is an ongoing process. The common outcome for airway macrophage in these situations is that they are less responsive to subsequent stimulation. A change in macrophage responsiveness is also evident following resolution of severe acute inflammation caused by viruses (Didierlaurent et al. 2008; Goulding et al. 2011) and allergens (Habibzay et al. 2012). The longevity of this change appears to depend on the severity of the prior inflammation and results in suppression of airway macrophage. This altered function also translates to humans since airway macrophage from a variety of lung inflammatory conditions are blunted in their responsiveness to further stimulation (Armstrong et al. 2009; Shaykhiev et al. 2009).

Viral infection, asthma, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and cystic fibrosis are all associated with recurring bacterial exacerbations leading to increased hospitalisations, deaths, and a significant economic burden. Despite the diversity in pathology, initial causative agent or process and the immune response induced, the bacterial species that emerge in each case are remarkably reproducible suggesting a common underlying process in each case. This process may be driven by a loss of regulation from epithelium, the clearance of apoptotic cells, or a change in the phenotype of repaired epithelium. Support from the latter comes from a recent ex vivo and in vitro study of the asthmatic epithelium where they demonstrate an intrinsically altered phenotype and aberrant inflammatory response to common environmental challenges compared with non-asthmatic epithelium (Hackett et al. 2011).

8.5 Conclusion

Airway macrophage have a markedly different phenotype to their counterparts from other more sterile sites of the body, both in terms of their expression of surface markers and response to inflammatory stimuli. These adaptations have led to a macrophage well suited to a unique microenvironment where a large amount of antigen is routinely breathed into the airway. Whilst it is critical to mount an immune response to airborne pathogens, inappropriate responses to all inhaled antigen would result in a constant state of inflammation in the lung. A high threshold of activation is achieved through the expression of a range of negative regulators such as CD200R, ST2L, and TREM-2 on macrophage, and their associated ligands on the epithelial surface, and serves to limit the responsiveness of airway macrophage. The resolution of both infectious and non-infectious inflammation in the airway leads to

a further increase in the expression of alveolar macrophage negative regulators. This innate imprinting by previous exposure to inflammatory stimuli effectively sets the threshold of macrophage activation.

Unlike other macrophage populations, airway macrophage display surface markers more akin to dendritic cells, such as CD11c. In contrast to antigen sampling in the intestine for example, which relies on transport of luminal antigen across the gut epithelium, airway macrophage reside in the lumen of the airspaces and therefore are the very first line of defence in the detection of pathogens. Due to this critical role, altering the activation threshold of airway macrophage will have a significant impact on susceptibility to respiratory infections and disease. Expanding our knowledge of these regulatory pathways will open new avenues for the immunomodulation of innate immunity in the airway and the treatment of respiratory diseases.

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Chapter 9
Microglial Ontogeny and Functions in Shaping Brain Circuits

Guillaume Hoeffel, Paola Squarzoni, Sonia Garel, and Florent Ginhoux

9.1 Introduction

Microglia are the resident macrophages of the central nervous system (CNS) and form a homogeneously distributed network throughout the brain and spinal cord (Fig. 9.1) (Lawson et al. 1990), accounting for about 5–20 % of adult CNS-cell population (Perry 1998). They belong to the glial cell compartment and their function is crucial to the maintenance of the CNS in both health and disease (Perry et al. 2010).

Two main functional aspects define microglia: immune defense and promotion of CNS homeostasis. As part of the innate immune system, microglia constantly sample their environment, scanning and surveying for signals of external danger (Lehnardt 2010), such as those from invading pathogens, or internal danger signals generated by damaged or apoptotic neurons (Bessis et al. 2007; Hanisch and Kettenmann 2007). Detection of such signals induces drastic changes in their behavior within the neuronal network. Microglia aim to both resolve the "danger" and, importantly, simultaneously moderate the potential damage that inflammatory events can inflict on the CNS, while supporting tissue repair and remodeling (Minghetti and Levi 1998). Alongside this complex immunological balancing act in the adult brain, microglia are emerging as crucial contributors to brain homeostasis

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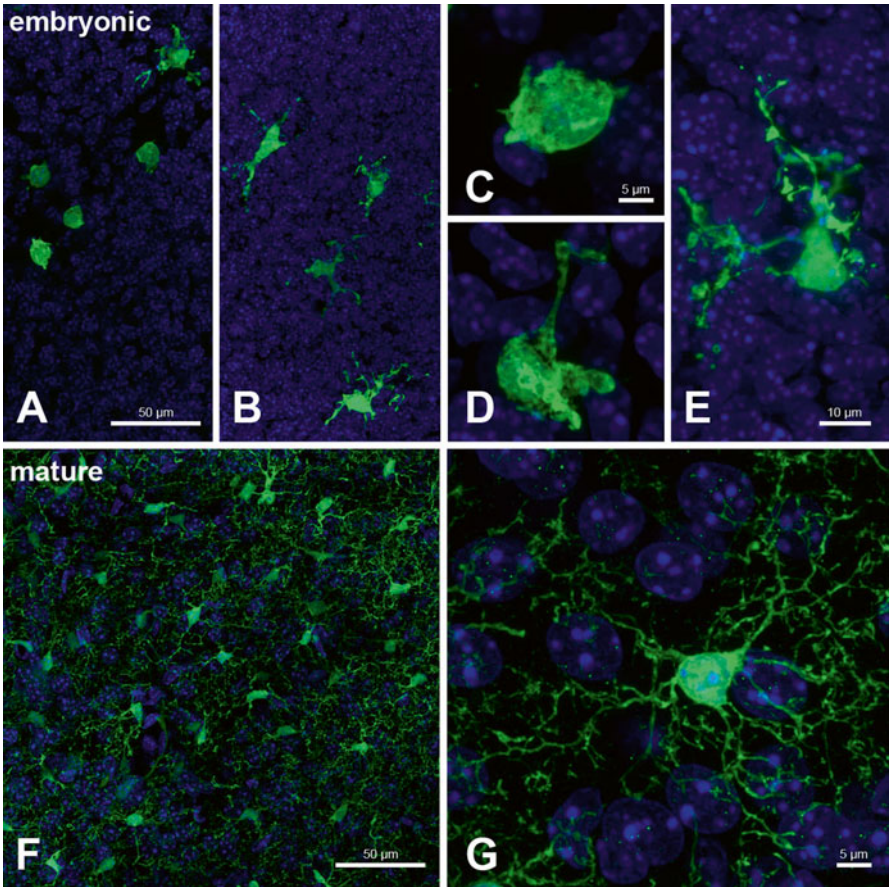

Fig. 9.1 (**a**–**e**) Images of microglial cells (green) in embryonic mouse brain (E14.5). Of note, depending on brain localization, the cells present different morphologies spreading from a round, amoeboid (**a**, **c**, **d**) to a ramified shape (**b**, **e**). (**f**, **g**) Images of microglial cells in mature postnatal mouse brain (P20). The cells appear uniformly distributed in the brain tissue and morphologically highly ramified, with very long and branched extensions. Pictures taken from *cx3cr1*$^{+/gfp}$ embryonic and mature mouse brains after GFP immunohistochemistry. Hoechst (blue) was used as nuclear staining

(Davalos et al. 2005; Nimmerjahn et al. 2005) through control of neuronal proliferation and differentiation, as well as influencing formation of synaptic connections (Graeber 2010; Hughes 2012). Recent imaging studies revealed dynamic interactions between microglia and synaptic connections in healthy brain that contributed to the modification and elimination of synaptic structures (Tremblay et al. 2010). Microglia also contribute to the remodeling of post-natal neural circuits as they have been recently shown to play a role in synaptic pruning (Paolicelli et al. 2011; Schafer et al. 2012). Thus, microglia occupy a central position in the defense and maintenance of the CNS, and as a consequence have attracted much interest as a target for the treatment of neurological and psychiatric disorders.

However, therapeutic exploitation of the microglial population has been held back by an incomplete understanding of their own origins, differentiation, and homeostasis. While microglia have been studied for decades, a long history of experimental misinterpretation meant that their true origins remained debated until recently. The finding that these cells in fact arise from yolk sac primitive macrophage progenitors that invade the brain rudiment at very early stages of embryonic development (Ginhoux et al. 2010, 2013; Schulz et al. 2012; Kierdorf et al. 2013) led to the discovery that embryonic microglia persist in the CNS into adulthood. Moreover, this early embryonic brain colonization by microglia is highly conserved across vertebrate species, suggesting that the embryonic microglial population is in fact essential for early brain development (Herbomel et al. 2001; Verney et al. 2010; Schlegelmilch et al. 2011; Swinnen et al. 2013), as well as adult brain homeostasis.

In this book chapter, we will present the latest advances in our knowledge on the origin, differentiation pathways, and homeostasis of the microglial population, which provide new insights into their roles in health and disease. We will also review the evidence for a microglial contribution to the development of the CNS and shaping of postnatal neuronal circuits. Finally, in light of these advances in our understanding, we will discuss the potential involvement of microglia in developmental pathologies of the CNS.

9.2 Nature of Microglia

9.2.1 Historical Perspectives

The recognition of a distinct microglial population within the CNS and how these cells originate during development have been a matter of debate for generations of researchers over the past 150 years. The first description of cells in the brain distinct from neurons came from the work of Virchow in 1856, who named them "nevernkitt," meaning nerve glue, and translated as neuroglia. It turned out decades later that the neuroglial population was comprised of two types of cell, astrocytes and oligodendrocytes, corresponding to the population we now know as macroglia (Rio-Hortega 1939). In the late nineteenth century, Franz Nissl described unique rod cells that he named "Stabchenzellen," as a "glial element with migratory, phagocytic and proliferative potential". In the early twentieth century, W. Ford Robertson introduced the term "mesoglia" to describe a mesoderm-derived phagocytic element in the CNS that had an origin distinct from that of neurons and neuroglia, which arise from the neuro-ectoderm. Santiago Ramon y Cajal later renamed the same cells the "third element of the nervous system" to differentiate them from neurons and neuroglia and stated again that they were of probable mesodermal origin. Del Rio-Hortega, a student of Ramon y Cajal, exploited silver staining techniques to further refine the description of the two types of non-neuronal cells contained in the third element of the nervous system as differing in origin, distribution, form, and function: the major population, called oligodendroglia, that lacked phagocytic activity, and

the minor population of ramified resting cells. This minor population was then clearly defined as the "third element of the CNS" with a mesodermal origin, containing phagocytic corpuscles and with migratory activity (Rio-Hortega 1932). Finally, Del Rio-Hortega introduced the term "microglial cell" to describe these cells as the non-neuronal, non-astrocytic third element distinct from neuroectodermal oligodendroglia or oligodendrocytes that constitute the macroglia (Rio-Hortega 1939) [For historic review see (Rezaie and Male 2002)].

9.2.2 Microglia as a Tissue Macrophage

Despite Del Rio-Hortega's seminal work, his theories were largely overlooked as, during his time, there was much support for the belief that microglia shared a neuroectodermal origin with the other glial cells—a belief that persisted well into the twentieth century. For example, Fujita and Kitamura proposed a common, matrix-derived progenitor for microglia, astrocytes, and oligodendrocytes (Fujita and Kitamura 1975), suggesting that microglia, as well as astrocytes, originated from neuro-ectodermally derived glioblasts (Kitamura et al. 1984). Even into the late 1990s, some studies still perpetuated the belief of a shared origin of astrocytes and microglia (Hao et al. 1991; Fedoroff et al. 1997).

However, other investigators, following Del Rio-Hortega's first hypothesis, presented evidence supporting a mesodermal origin of microglia: first by coupling light/electron microscopy and immunohistochemistry, which allowed identification of parallel morphological features of macrophages and microglia at various stages of development (Murabe and Sano 1982), and second by demonstrating that microglial cells were recognized by antisera raised against monocyte/macrophage antigens (Hume et al. 1983). Phenotypic homologies between monocytes, macrophages, and microglia were validated by immunohistochemical studies that reported expression of macrophage markers, including F4/80, Fc receptor, and CD11b, in mouse (Perry et al. 1985) and in human microglia (Akiyama and McGeer 1990). Finally, a seminal genetic study revealed that mice deficient for PU.1, a crucial transcription factor for myeloid cell development, were also devoid of microglia (McKercher et al. 1996; Beers et al. 2006). This unequivocally established the myeloid nature of the microglial population and simultaneously implied that these cells might be ontogenetically related to macrophages.

9.2.3 Markers of Microglia

As the tissue-resident macrophage of the CNS, murine microglia express several classical macrophage markers including the CSF-1 receptor (CSF-1R, CD115), the integrin CD11b, the surface glycoproteins F4/80, CD200R, the surface enzyme Tyrosine-protein phosphatase non-receptor-type substrate or CD172α, the

Table 9.1 Surface markers that distinguish Microglia from Monocytes and Perivascular Macrophages

Cells	Microglia	Monocyte (all)	Perivascular macrophage
Markers	$CD45^{lo}$	CD45	CD45
	CD11b	CD11b	CD11b
	CD115	CD115	CD115
	F4/80	F4/80	F4/80
	$CX3CR1^{hi}$	Cx3CR1	CX3CR1
	CD64	*Inflammatory monocyte*	
	Iba1	$Ly6C^{hi}$	MOMA-2
	Siglec H	CCR2	ED2 (CD163)
	CD172a	CD62L	
		Patroling monocyte	
		CD11a	
References	Jung et al. (2000)	Geissmann et al. (2003)	Mendes-Jorge et al. (2009)
	Gautier et al. (2012)		Dijkstra et al. (1985)

fractalkine receptor CX3CR1 (Fig. 9.1), and the calcium-binding protein Iba-1 (Table 9.1). However, only few markers have been described to enable their specific identification. The main exploitable difference between microglia and other tissue macrophages is the lower microglial expression of the pan-hematopoietic marker CD45, which permits their discrimination from monocytes, and CD163 that identify perivascular macrophages in the steady state (Dijkstra et al. 1985; Serrats et al. 2010).

While fractalkine receptor expression is shared by microglia, some other macrophages and monocytes, experiments in *CX3CR1*$^{gfp/+}$ knock-in reporter mice revealed that microglia express the highest levels among these cell types (Jung et al. 2000). Fractalkine (CX3CL1) is expressed by specific neurons, and loss of its receptor in the homozygote *CX3CR1*$^{gfp/+}$ knock-in reporter mice clearly demonstrates the importance of this axis in the CNS as we will discuss later: in this model, insertion of the GFP reporter gene generates a null allele which results in neurotoxicity (Cardona et al. 2006) and defective apoptotic clearance and synapse remodeling during development (Paolicelli et al. 2011; Wolf et al. 2013).

9.2.4 Differentiation of Microglia

The fact that microglia express CSF-1R (Sawada et al. 1990) is further evidence of their relationship to other macrophages and of their myeloid nature. Colony-stimulating factor-1 (CSF-1) is a key growth factor for the generation, differentiation, maintenance, and function of tissue macrophages (Guilbert and Stanley 1980; Wiktor-Jedrzejczak et al. 1990) and its effect is mediated by the CSF-1 receptor tyrosine kinase CSF-1R (Dai et al. 2002). Mice that carry a spontaneous recessive inactivating mutation in the gene encoding CSF-1 (*Csf1*$^{op/op}$), or have been rendered

deficient for its receptor CSF-1R (CSF-1R KO), lack several macrophage populations across various tissues (Yoshida et al. 1990; Dai et al. 2002). We recently showed that microglia are one of those populations. These cells were absent in embryos, and in the CNS of newborn and adult CSF-1R KO mice, suggesting that the development of microglia, as other macrophages, is strongly dependent on the CSF-1R pathway (Ginhoux et al. 2010). In agreement with such observations, mice deficient for PU.1, a transcription factor that controls CSF-1R expression, are also devoid of microglia and other macrophage populations (McKercher et al. 1996; Beers et al. 2006). While the importance of CSF-1R expression for microglial development is obvious, it is currently unclear whether CSF-1R is also required to maintain microglial homeostasis in vivo in the adult.

Interestingly, in contrast to CSF-1R KO mice, *Csf1*$^{op/op}$ mice exhibit relatively normal microglial development, with just a 30 % reduction in population numbers (Ginhoux et al. 2010). These observations implied the existence of an alternative ligand for CSF1-R that could compensate for the absence of CSF-1 and enable the maintenance of microglia in vivo. Consistent with this possibility, interleukin-34 (IL-34) has recently been identified as an additional ligand for CSF1-R in mice and humans (Lin et al. 2008). IL-34 binds CSF1-R at different regions (Chihara et al. 2010) and with a higher affinity than CSF-1 in vitro and is more conserved in mammalian and avian species than CSF-1, hinting that IL-34 might play an important role in macrophage homeostasis (Garceau et al. 2010). Expression of IL-34 in a CSF-1-dependent manner rescued the phenotype of CSF-1-deficient mice (Wei et al. 2010), suggesting that CSF-1 and IL-34 comparably regulate CSF-1R signaling. IL-34 itself enhances the neuroprotective effects of microglia in vitro (Mizuno et al. 2011), and is expressed at high levels in the brain of adult mice, suggesting a role for IL-34-mediated CSF-1R signaling in these tissues (Wei et al. 2010). Recently, two groups generated IL-34 KO mice using a similar strategy (Wang et al. 2012; Greter et al. 2012) and reported that in the brain, the deficiency of IL-34 led to a significant decrease but not total in microglial cell numbers. Wang et al. also reported dramatic defects in neonatal microglia as illustrated by the almost complete lack of microglial cells in IL-34 KO newborns. However, Greter et al. did not observe such a reduction of microglia in IL-34-deficient neonates and hypothesized that the expression of CSF-1 in the CNS parenchyma compensated for the loss of IL-34. The exact roles of the two CSF-1R ligands in microglial development and homeostasis in the adult, and whether they have the same or different roles during development in embryos, have yet to be precisely determined. Interestingly, IL-34 possesses a spatio-temporal expression pattern that differs from that of CSF-1, permitting complementary activation of the CSF-1R in both embryonic and adult tissues (Wei et al. 2010). Finally, an alternate receptor for IL-34 was recently identified: the receptor-type protein tyrosine phosphatase zeta (PTP-ζ) is a cell-surface chondroitin sulfate proteoglycan that is primarily expressed on neural progenitors and glial cells, suggesting that IL-34 may have a wider repertoire of effects within the CNS than previously appreciated (Nandi et al. 2013).

Others molecules has been shown to regulate microglia homeostasis. While microglial differentiation and homeostasis rely on CSF-1R signaling, their activation

is tightly regulated by receptors and co-receptors for inflammatory signals, including the adaptor protein DAP12 and TREM2 (triggering receptor expressed on myeloid cells-2). In vitro, DAP12 controls proliferation and survival of macrophages stimulated with CSF-1 (Otero et al. 2009), while in vivo in older mice deficient in DAP12, fewer microglia are present in defined regions of the CNS (Otero et al. 2009), which suggests a role for DAP12 in the long-term homeostasis of microglia. Humans with mutations in either the gene for DAP12 or TREM2 develop Nasu-Hakola disease, characterized by bone cysts, bone fractures, and psychotic symptoms leading to severe neurodegeneration and encephalopathy (Paloneva et al. 2000).

9.3 The Embryonic Origin of Microglia

The evident phenotypic similarities between microglia and other macrophage populations led to ready acceptance of their myeloid origin, although the true identity of microglial progenitors remained controversial until recently. Initial studies described the presence of microglial cells during early development, suggesting that microglia arise from embryonic progenitors. These progenitors were first proposed by Del Rio-Hortega to be meningeal macrophages infiltrating the brain during early embryonic development. However, Del Rio-Hortega also believed that microglia could also be derived from blood monocytes. Monocytes are indeed recruited to the neonatal and adult brain, in the latter case most often under inflammatory conditions, where they can differentiate into microglia-like cells. These observations long supported the prevailing viewpoint that blood-circulating monocytes represented microglial progenitors, replacing those seeding the brain during embryonic development. In fact, until recently, the most consensual hypothesis was that embryonic and perinatal hematopoietic waves of microglial recruitment and differentiation occurred in the CNS. Here, we will describe the latest advances in understanding of the origin of microglia.

9.3.1 Early Development

When Del Rio-Hortega first described microglia, he also proposed that they might initially arise in the early stages of development from mesodermal cells of the pia mater, the innermost layer of the meninges (the membranes surrounding the CNS). He reported the "migration of embryonic corpuscles [with morphological similarity to lymphocytes] from the pia into the nerve centres" but simultaneously proposed that "microglia may eventually arise from other related elements, chiefly the blood mononuclears," based on the similarities in morphology and phagocytic activity between microglia and monocytes (Rio-Hortega 1939). These two statements were the founding of the "Origin of Microglia" controversy that was to last for the next 50 years.

Perry et al. were among the first to begin to resolve the issue in their seminal immunochemistry study. They exploited macrophage markers expressed by microglia to show that, as early as murine embryonic day 16 (E16), macrophage-like cells, likely to be microglial progenitors, invade the brain parenchyma and become localized in hot spots where they differentiate through a series of transitional forms to finally become ramified microglia (Perry et al. 1985) (Fig. 9.1). Similarly, in the rat, amoeboid microglial cells expressing monocytic/macrophagic markers have been described as early as E12 in the neuro-epithelium (Morris et al. 1991; Wang et al. 1996). Further support for the identification of these embryonic cells as microglial progenitors was provided by in vitro demonstration of their high proliferative potential upon mitogenic stimulation (Alliot et al. 1991).

Similarly, in human fetuses, microglia-like cells with a range of morphologies can be detected as early as 13 weeks of estimated gestational age (EGA) (Hutchins et al. 1990). However, it appears that maturation of the microglial compartment is ongoing throughout the majority of gestation: colonization of the spinal cord begins at around 9 weeks, the major influx and distribution of microglia commences at about 16 weeks, and ramified microglia take up to 22 weeks to become widely distributed within the intermediate zone (Rezaie and Male 1999; Rezaie et al. 2005). In fact, it is only close to term, at 35 weeks, that well-differentiated microglial populations can be detected within the developing human brain (Esiri and Morris 1991) [for review (Rezaie 2003; Rezaie et al. 2005; Verney et al. 2010)].

Altogether, these seminal studies suggested that microglia derive from embryonic hematopoietic precursors that seed the CNS prior to birth and, more importantly, before the onset of bone marrow (BM) hematopoiesis, raising the hypothesis that microglia derive from unique embryonic precursors not found in the BM. However, two questions remained: what was the exact tissue of origin and developmental lineage of the precursors that migrate to the CNS to give rise to the "first" endogenous wave of microglia, and what happened to this initial embryonically derived microglial population during postnatal development and adulthood?

9.3.2 Embryonic Hematopoiesis

The sites of origin of hematopoietic progenitor cells have been a subject of long-standing debate and intense investigation. Embryonic hematopoiesis takes place in different tissues during development, with the major site of hematopoiesis shifting from one organ to another in a temporal and spatial manner, adding another level of complexity to the identification of microglial precursors. Among these tissues, three are considered of major importance in the embryo: the extra-embryonic yolk sac (YS), the paraaortic splanchnopleura (PSp), that will become the aorta, gonads, and mesonephros (AGM) region, and the intra-embryonic fetal liver (Tavian and Peault 2005; Orkin and Zon 2008). Hematopoietic cells arise directly from mesodermal precursors within YS, the PSp, and the AGM (Fig. 9.2). Other hemogenic endothelium sites include the umbilical and vitelline arteries, where clusters of

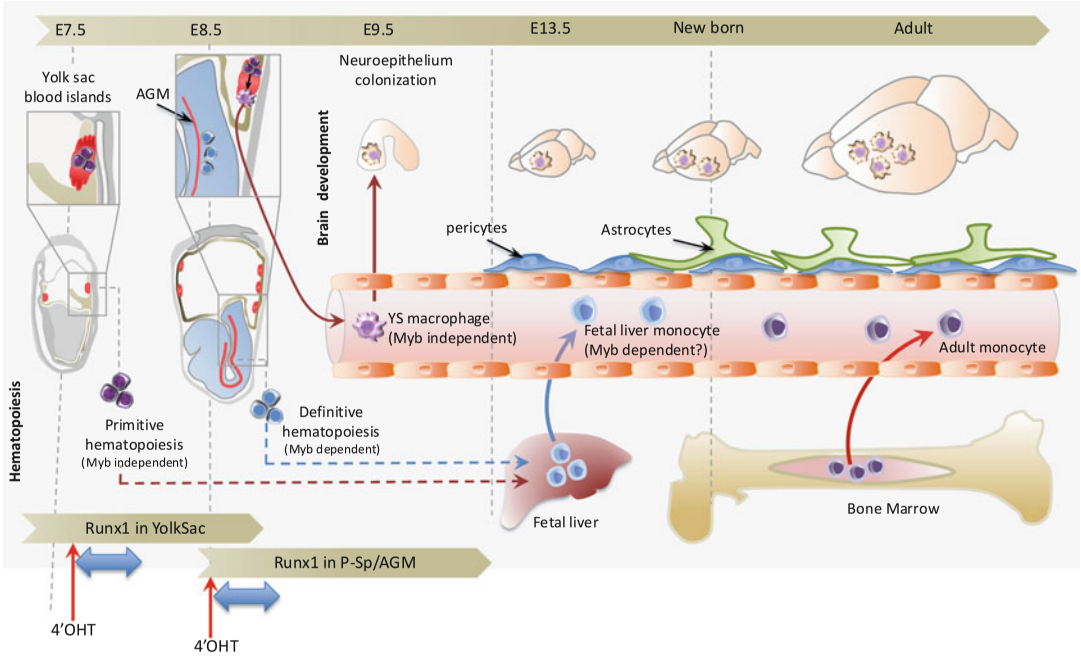

Fig. 9.2 Brain development and microglia ontogeny. The wave of primitive progenitors generated in the yolk sac (YS) blood islands give rise to primitive macrophages at E8.0/E9.0. They spread into the embryos at the onset of blood circulation established at E8.5 and colonize the neuro-epithelium from E9.5 to give rise to embryonic microglia. The blood brain barrier starts to form from E13.5 and may isolate the developing brain from the contribution of fetal liver and later of bone marrow hematopoiesis. Embryonic microglia expand and colonize the whole CNS and persist until adulthood. In parallel, definitive hematopoiesis occurs in the aorta-gonads-mesonephros (AGM) at E8.5 and gives rise to progenitors that colonize the fetal liver. Progenitors from the YS can also contribute to the fetal liver hematopoiesis during middle embryonic development. Based on the specific temporal Runx1 expression during development and using the Runx1-Mer-Cre-Mer mice, it is possible to selectively tag either the YS or AGM progenitors by injecting hydroxytamoxifen (4′OHT) at E7.5 or E8.5 respectively

hematopoietic cells appear in the lumen (Yokomizo and Dzierzak 2010), but their contribution to definitive hematopoiesis is uncertain. Other hematopoietic organs, including the fetal liver, spleen and BM are subsequently colonized by hematopoietic progenitor entering the blood circulation (Fig. 9.2).

In mice, the first site of hematopoiesis is the extra-embryonic YS, which is the membrane surrounding the embryo. In the YS, at around E7.0, shortly after the onset of gastrulation, the mesodermal layer develops into structures referred to as blood islands that are responsible for the first wave of hematopoiesis (Fig. 9.2). YS hematopoiesis is often termed "primitive hematopoiesis" as it predominantly produces nucleated erythrocytes and primitive macrophages (Moore and Metcalf 1970; Palis et al. 1999; Bertrand et al. 2005). These macrophages first appear in the YS blood islands at E8.5-E9.0 and their pattern of differentiation is unique in the sense that they do not go through the monocytic intermediate stage seen in adult macrophages (Takahashi et al. 1989). YS-derived primitive macrophages spread into the embryo proper through the blood as soon as the circulatory system is fully established (from E8.5 to E10) (McGrath et al. 2003), and migrate to various tissues including the brain. Importantly, this process happens before the onset of monocyte production by the fetal liver which starts around E11.5-E12.5 (Naito et al. 1990). A defining feature of these primitive macrophages (compared to adult tissue macrophages) is that they exhibit a high proliferative potential in the YS and in every tissue they colonize (Takahashi et al. 1989; Sorokin et al. 1992; Naito et al. 1996; Lichanska and Hume 2000).

At E8.5–E9.0, just after the initiation of YS hematopoiesis, the intra-embryonic PSp mesoderm gives rise to the fused aorta surrounded by gonads and mesonephros, a region referred to as the AGM (aorta, gonads, and mesonephros), wherein a new wave of hematopoietic progenitors is generated within the embryo proper (Godin et al. 1993; Medvinsky et al. 1993). The hematopoietic stem cells (HSC) generated within the AGM will establish definitive hematopoiesis (Orkin and Zon 2008) with multilineage hematopoietic potential (Fig. 9.2). Around E10.0–E10.5, YS- and AGM-derived hematopoietic progenitor cells colonize the fetal liver (Kumaravelu et al. 2002) that becomes active as a hematopoietic site with HSC activity around E11.0–E11.5. The fetal liver serves as the major hematopoietic organ after E11.5, generating all hematopoietic lineages, including monocytes (Naito et al. 1990). Near the end of gestation (E15.0–E16.0), the presence of HSC in fetal liver and spleen regresses, concomitant with the migration of these cells to the BM that will be the principal hematopoietic organ through adult life.

A recent study highlighted further differences between primitive and definitive hematopoiesis, showing that the latter relies on the transcription factor Myb, while YS-derived macrophages are Myb-independent, but PU.1 dependent (Schulz et al. 2012) in contrast to a previous study that reported that mice with null mutation in PU.1 had normal numbers of c-fms-positive phagocytes at E11.5 (Lichanska et al. 1999). This further underlines the fact that YS-derived macrophages constitute an independent lineage, distinct from the progeny of definitive hematopoietic stem cells.

Embryonic human and mouse hematopoietic systems are organized similarly. Human hematopoiesis also begins in the YS blood islands around day 18–19 of

EGA, and lasts from weeks 3 to 6. Similarly, primitive YS-derived stem cells are limited to myelo-erythroid development. HSC generation in the AGM occurs at weeks 5–7, hematopoiesis then moves transitorily to the fetal liver at around 4–5 weeks EGA until 22 weeks EGA, before being definitively established in the BM at approximately 10.5 weeks EGA (Tavian and Peault 2005).

9.3.3 The Yolk Sac Origin

As discussed before, microglia-like cells are present in the developing brain before birth and preceding the initiation of BM hematopoiesis. Therefore, it is likely that microglial progenitors arise from either extra-embryonic YS or the intra-embryonic fetal liver, or possibly both. Ashwell was the first to report the presence of round and amoeboid microglial cells in the fetal mouse cerebellum (Ashwell 1990) and then in rat forebrain (Ashwell 1991) as early as E11.0. Sorokin soon after detected macrophage-like cells and their precursors in blood vessels and the embryonic mesenchyme in rat embryos from E10.5, and noted that the developing brain was the first organ to be colonized (Sorokin et al. 1992). These data were suggestive of a YS origin of microglial cells, as their appearance in the brain preceded the initiation of fetal liver hematopoiesis. Interestingly, cells with the capacity to differentiate into microglia-like cells in vitro (expressing Mac-1, Mac-3, F4/80, and Fc antigens, with a macrophagic morphology and ultrastructure) can be detected in the developing murine neuro-epithelium at E9.0, suggesting that this is the earliest developmental stage at which seeding of cells with myeloid features occurs in the brain (Alliot et al. 1999). Later reports confirmed the presence of amoeboid cells expressing macrophage (Alliot et al. 1999; Ginhoux et al. 2010) and microglial markers (Chan et al. 2007; Mizutani et al. 2012) at this developmental period in both the cephalic mesenchyme and the neuro-epithelium. This further supported the idea that the YS contributes to microglial genesis, as at this early stage the YS is the only site of hematopoiesis.

However, the evidence for a YS origin of microglial progenitors was, at first, mixed. Initially, data from one of the aforementioned in vitro studies were interpreted to support the hypothesis that these macrophage-like cells that will give rise to microglia originated from the neuro-ectoderm (Hao et al. 1991). However, Takashi and Naito drew a different conclusion after they described the first emergence of immature macrophages within blood islands of embryonic YS at E9.0 in both mouse (Takahashi et al. 1989) and rat (Takahashi and Naito 1993). Following the establishment of fetal blood circulation, these cells colonize the embryonic tissues, including the brain rudiment. By [3H]thymidine autoradiography, YS macrophages were shown to possess high proliferative potential, which suggested that the immature fetal macrophages were in fact primitive macrophages from the YS (Takahashi and Naito 1993). Alliot's work later conclusively documented the presence of potential microglial progenitors in the YS and then the brain rudiment, with their numbers increasing dramatically from E9.0–E9.5 until around 2 weeks after birth (Alliot et al. 1999).

Interestingly, the YS derivation of microglia appears to be highly conserved across vertebrate species including zebrafish (Herbomel et al. 2001) and in avians (Cuadros and Navascues 2001). However what differs in mice is the requirement for a functional blood circulation for the spreading of YS macrophages into the embryo proper. In zebrafish, YS macrophages directly invade the whole cephalic mesenchyme and then populate epithelial tissues including the brain, while other macrophages enter the blood circulation (Herbomel et al. 1999). In mouse embryos, a clear requirement for the circulatory system is observed as E9.5–E10.5 Ncx-1$^{-/-}$ embryos, which have no functional blood circulation (Koushik et al. 2001), also lack microglial progenitors (Ginhoux et al. 2010), as well as other fetal macrophages, despite normal YS hematopoiesis (Ginhoux et al. 2010).

There is some evidence that a similar pattern of events occurs in humans, where, from 4.5 weeks gestation, amoeboid microglial cells (characterized by the expression of Iba1, CD68, CD45, and MHC-II) enter the cerebral wall from the ventricular lumen and the leptomeninges (Monier et al. 2007). In the YS and mesenchyme at 4–6 weeks after fertilization, two populations of cells with a dendritic morphology can be distinguished: a majority that express monocyte/macrophage-associated markers but no detectable HLA-DR antigen, and a minority constitutively expressing MHC class II (HLA-DR and -DP), but no monocyte/macrophage-associated markers (Janossy et al. 1986). The emergence of this heterogeneity preceded the formation of both thymus and BM, suggesting the independent development of these macrophage populations (for review Verney et al. 2010).

The overall conclusion of the studies in rodents, humans, and other species is that microglia derive from the YS macrophages that seed the brain rudiment during early fetal development (Fig. 9.2). However, these reports could not exclude the possibility that other progenitors might supersede the YS contribution with time or under certain physiological conditions. In fact, some data continued to emerge that suggested a requirement for the contribution of blood-borne cells, such as monocytes, to both generate the post-natal microglial compartment, and to maintain it into adulthood.

9.4 Post-natal Hematopoietic Contribution to the Microglial Compartment

9.4.1 Contribution in Steady State

Shortly after birth, the microglial population expands so dramatically (Alliot et al. 1999; Tambuyzer et al. 2009) that it was thought that the proliferation of embryonic microglial cells alone could not account for the steep rise in numbers, and so there must be a fresh influx of cells from another compartment. As initially suggested by Del Rio-Ortega, blood monocytes were believed to invade the CNS in the perinatal period and give rise to microglia, replacing the embryonic microglial cells. Several studies supported this seminal hypothesis, notably an early report

(Ling 1976) where round, amoeboid, phagocytic cells were seen in rat corpus callosum during the first few days of life and then disappeared coincident with the appearance of ramified microglia. These cells were typical macrophages, but some displayed features of monocytes, while others appeared to be transitional between the two types. The authors of this study concluded that circulating monocytes enter the developing brain, differentiate first into amoeboid microglia, and then secondly become ramified microglia (Ling 1976). Subsequent studies gave neo-natal rats an intraperitoneal pulse of [3H]thymidine to allow tracking of labeled blood cells by autoradiography. Labeled immature amoeboid cells were detected in the corpus callosum a few hours after thymidine administration, while the majority of newly ramified microglia were labeled 1 week later. These observations implied that labeled microglial cells must therefore have come from the transformation of immature amoeboid cells that had acquired the tracer earlier (Imamoto and Leblond 1978). Monocyte tracking studies employing carbon particle labeling strategies detected carbon in amoeboid cells of the corpus callosum and then on ramified microglial cells, suggesting again that blood monocytes migrated to the corpus callosum and differentiated into microglial cells via an amoeboid stage (Ling 1979; Ling et al. 1980; Leong and Ling 1992). However, while such data did show that circulating blood monocytes could enter the CNS soon after birth, perhaps in a specific site, it is important to note that these studies were predominantly descriptive and unable to address the relative contributions of post-natal monocytes versus embryonic progenitors to the adult microglial population. In fact, the authors had themselves thought that such events might be infrequent (Ling et al. 1980).

Additional evidence for the lack of significant contribution of monocytes or other BM-derived progenitors to the adult microglial pool came from prolonged experiments performed in parabiotic mice, where two adult congenic mice undergo surgery to physically link their circulatory systems. A few weeks after the initiation of parabiosis, leucocyte populations in the blood comprised cells from both animals (Liu et al. 2007). However, while T cell and B cell mixing reached a 50 % equilibrium, monocyte mixing was significantly lower with only 15–30 % chimerism in both animals, which likely reflected the short half-life of monocytes in the blood. Nevertheless, if monocytes were differentiating into microglia in the steady state, a contribution from the non-host parabiont would be expected to occur at a level comparable to that of monocyte mixing level. In reality, even after up to 12 months of parabiosis, microglia failed to mix, clearly illustrating the absence of contribution of BM-derived cells to the CNS microglial population (Ajami et al. 2007; Hashimoto et al. 2013). This distinction between the mechanisms maintaining blood monocyte versus microglial populations was further exemplified in parabionts where one animal lacked functional CCR2, and therefore was defective in monocyte emigration from the BM into the circulation (Serbina and Pamer 2006). In this case, over 70 % of monocytes detected in the CCR2 KO mice were from the other wild-type parabiont. Despite this, chimerism of microglia remained negligible even after 1 year of parabiosis (Hashimoto et al. 2013). Altogether, in these models of steady state homeostasis, it seems that maintenance and local expansion of microglia are solely dependent on the self-renewal of CNS-resident cells. Interestingly, this now also

appears to be the case for other tissue-resident macrophage populations which have been recently shown to self-maintain locally throughout adult life with minimal contribution from circulating monocytes (Yona et al. 2013; Hashimoto et al. 2013).

However, the fact that monocytes have conclusively been shown to be able to contribute to the microglial population in adult animals should not be overlooked. One study in particular transplanted wild-type BM into PU.1 KO neonatal mice, which lack embryonic microglia, and observed de novo generation of the full microglial compartment (Beers et al. 2006). Therefore, it must be concluded that, at least under exceptional circumstances such the complete absence of endogenous embryonic microglia, some BM-derived cells had the capacity to infiltrate the CNS and assume the morphology and phagocytic capacity of microglia. What remains to be understood are the physiological circumstances in which this process is likely to be significant in adults, and whether these BM-derived microglia are fully functionally competent in situ.

9.4.2 Contribution During Inflammation

Although the monocyte-to-microglia path may exist in adult brain, it is unlikely to be a significant source for maintaining the microglial population in the steady state. However, this might change during CNS inflammation or disease (Vallieres and Sawchenko 2003; Mildner et al. 2007). In fact, in response to CNS inflammation and damage, an increase in microglial number is often observed, a phenomenon called reactive microgliosis, which has become a hallmark of many CNS pathologies (Fig. 9.3). However, it remained to be elucidated whether such increases in number rely on local expansion of mature microglia or are achieved by recruitment of blood precursors such as monocytes.

Two recent studies have clarified the contribution of blood monocytes to microglia in experimental models of CNS pathologies. Both revealed that the irradiation regimen used to prepare recipient animals for BM transplants (allowing the distinction of donor blood contribution versus host microglia as microglia are radioresistant, see Ginhoux et al. 2013) is necessary for the recruitment and differentiation of monocytes into microglia (Ajami et al. 2007; Mildner et al. 2007). Mildner showed that recipient mice in which the CNS was shielded to protect from the irradiation and associated release of pro-inflammatory cytokines and chemokines did not experience a significant invasion of BM-derived cells into the brain, in contrast to the unshielded mice (Mildner et al. 2007). Beyond the irradiation issue, these data also suggest that microglial engraftment from the blood requires pre-conditioning of the CNS that likely disrupts the blood–brain barrier (BBB). Additional clarity came from experiments in parabiotic mice that enabled study of the turnover of hematopoietic cells for prolonged periods without the need for irradiation (Ajami et al. 2007). Ajami used such mice to show that in contrast to what was observed in irradiated and transplanted mice, there was no microglial progenitor recruitment from the circulation in either denervation or CNS neurodegenerative disease.

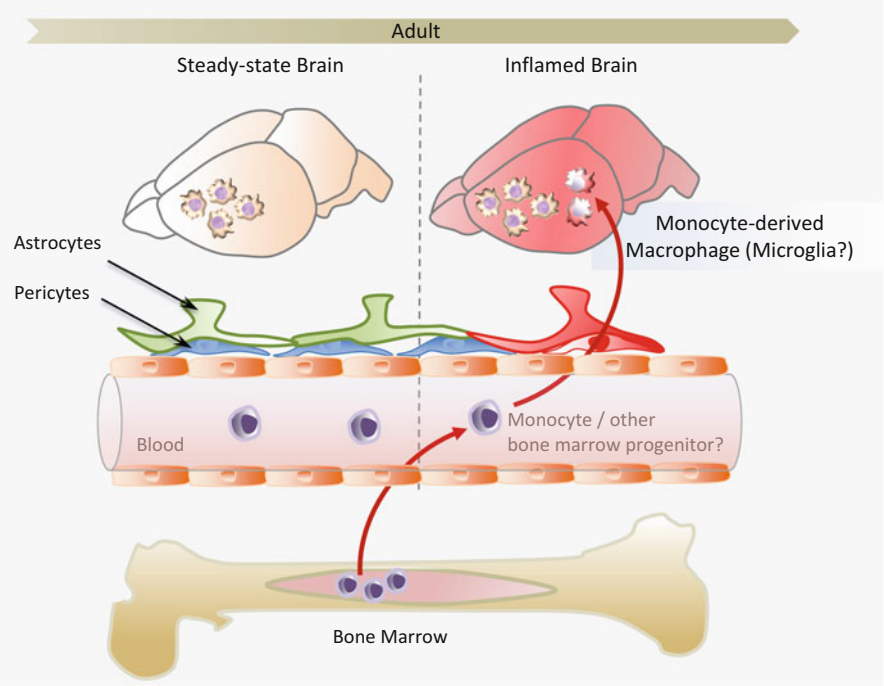

Fig. 9.3 Brain homeostasis at steady state or during inflammation. Embryonic microglia will maintain themselves until adulthood, via local proliferation during late gestation and post-natal development, although proliferative activity is diminishing with time being at very low levels in adult CNS. Under certain inflammatory conditions, the recruitment of monocytes or other bone marrow-derived progenitors can supplement the microglial population to some extent. However, we do not understand yet whether these cells persist and become integrated in the microglial network, or are a temporary addition to the endogenous population

Interestingly, with this parabiotic model, in the context of irradiation of one parabiont, no further contribution from the other parabiont was detected, contradicting the results of Mildner. However, Ajami further clarified that although irradiation is required for donor cells to engraft, it is not sufficient: another important, but often overlooked, requirement is the artificial introduction of a critical number of donor BM cells into the blood circulation (where they are not normally found). This, in conjunction with the inflammation of the BBB caused by irradiation, creates the unique non-physiological situation that is required for the monocyte-to-microglia pathway to prevail (Diserbo et al. 2002; Li et al. 2004; Capotondo et al. 2012). Taking this work further, the same group recently used a similar approach combining parabiosis and myelo-ablation to show that recruited monocytes do not persist in the CNS, and therefore even under these specific conditions do not finally contribute to the resident microglial pool. However, recruited monocytes do contribute to the severity of disease in multiple sclerosis and the experimental mouse model of autoimmune encephalitis (Ajami et al. 2011). In conclusion, parabiotic mice

provided, for the first time, unequivocal evidence that the microglial population during the steady state is able to maintain itself throughout adult life by local renewal, independent of circulating precursors. Conversely, in transplant models, which are perhaps not so much reflections of normal physiology, a fraction of microglia can arise temporarily from adult BM (Fig. 9.3).

9.5 Persistence of Microglia from a Unique Wave of Embryonic Progenitors

What remained unclear in the field, however, was the relative contribution of embryonic and post-natal hematopoietic progenitors to the steady state microglial population in adults: are the embryonic microglia responsible for maintaining the adult pool or do embryonic and adult microglia in fact have different origins? Although it could have been inferred from the previous aforementioned studies that showed that the maintenance and local expansion of microglia are independent of the contribution of peripheral blood/BM progenitors that embryonic microglia is mainly responsible for maintaining the adult pool. Notably, one of the studies already discussed, from De Groot, had implied that embryonic microglia were the sole contributors to the adult microglial pool. This study observed that donor BM cells failed to contribute to the adult microglial population in a model of newborn transplantation, and concluded therefore that the adult microglial population was totally independent of post-natal BM-derived circulating precursors from birth onward (de Groot et al. 1992). Recently, we revisited their experiments with a more quantitative aim and found that while most circulating leucocytes were of donor origin, the majority of microglia remained of host origin for more than 3 months after transplantation, confirming that post-natal hematopoietic precursors, including monocytes, likely do not contribute to the adult microglial population (Ginhoux et al. 2010).

We employed a more advanced technique of YS progenitor fate mapping to definitively answer the question of the origin of microglia. Our fate mapping mouse model expresses a fluorescent protein (eYFP) exclusively in YS progenitors and their progeny, which include YS macrophages. Briefly, this mouse model expresses a tamoxifen-activated MER-Cre-MER recombinase gene under the control of one of the endogenous promoters of the runt-related transcription factor 1 (Runx1) locus (Samokhvalov et al. 2007) (Fig. 9.2). When crossed with a Cre-reporter mouse strain, recombination can be induced in embryos by a single injection of 4-Hydroxytamoxifen (4′OHT) into pregnant females. Active recombination in these knock-in mice occurs in a short time frame that does not exceed 12 h post-injection, and leads to irreversible expression of eYFP in Runx1$^+$ cells and their progeny (Samokhvalov et al. 2007).

Although both YS and fetal liver hematopoietic progenitors express Runx1, YS progenitors are the only Runx1$^+$ cells present at E7.5 and so injection of tamoxifen at this time specifically and irreversibly tags YS progenitors and their progeny but not fetal liver-derived progeny (Fig. 9.2). In contrast, injection of tamoxifen at E8.5 or later will favor the tagging of AGM-derived hematopoietic progenitors and not

the YS progenitors (North et al. 1999; Samokhvalov et al. 2007) (Fig. 9.2). We can use this model to accurately ask about the origins of different cell types; for example, in the case of microglia, if they are predominantly derived from YS-tagged progenitors, they should express eYFP in the adult CNS when 4′OHT is injected at E7.5 and not at E8.5. In contrast, circulating leukocytes, including monocytes, which derive from AGM hematopoietic progenitors, should express eYFP when 4′OHT is injected at E8.5 instead of E7.5. In addition, if the microglial population does predominantly derive from YS progenitors without a significant contribution from fetal liver- or BM-derived hematopoiesis, they should be tagged at a higher level (when 4′OHT is injected at E7.5 and not at E8.5) than circulating leukocytes, which derive predominantly from mature hematopoiesis. To test this hypothesis, we injected the mice with tamoxifen at closely spaced time points of gestation and compared the number of eYFP-tagged microglia and circulating monocytes in the mice as they grew into adults. Strikingly, the relative number of tagged microglia in mice injected at E7.25 was much greater than that of blood monocytes or other circulating leukocytes (Ginhoux et al. 2010). In contrast, the relative number of tagged microglia in mice injected from E8.0 onwards decreased dramatically, reaching undetectable levels in mice injected as close as E8.5, while the relative number of eYFP$^+$ leukocytes, including monocytes, increased progressively in adult blood. This opposing pattern of recombination in microglia compared to circulating leukocytes strongly supports the idea that the major contribution to microglial numbers comes from YS progenitors, and formally excludes the contribution of definitive hematopoiesis. Altogether these results establish that microglia originate from E7.0–E7.5 Runx1 YS-derived hematopoietic progenitors, with little, if any, contribution from hematopoietic progenitors arising later in embryonic development. Recent studies have confirmed our findings (Schulz et al. 2012; Kierdorf et al. 2013), in particular, the latest study from Kierdorf refined the characterization of the YS precursors that give rise to microglia and identified them as early E8 primitive uncommitted F4/80$^-$ c-kit$^+$ erythromyeloid YS precursors which develop into CD45$^+$ckit^{lo}F4/80$^-$CX3CR1$^-$ myeloid progenitors before their maturation and migration into the developing brain as CD45$^+$c-kit$^-$CX3CR1$^+$ cells (Kierdorf et al. 2013).

These studies conclusively demonstrated that primitive macrophages are the embryonic source of the steady state adult microglial population. This was particularly interesting as it implied that microglia not only have a unique functional specialization within the CNS, but also a unique origin, arising from YS progenitors that maintain themselves by proliferating in situ throughout adulthood (Fig. 9.2). Beyond the case of microglia, it also provided startling evidence for a broader conclusion that primitive macrophages are the ultimate source of a functional immune compartment, which persists throughout adulthood. However, the case of microglia seems to be unique, as other fetal macrophage populations in the embryo will mostly be replaced by fetal liver-derived monocytes that seed the tissues later and differentiate into macrophages, as we recently showed for Langerhans cells (Hoeffel et al. 2012). Lack of differentiation of fetal liver-derived monocytes into microglial progenitors could result either from their lack of intrinsic differentiation potential or lack of access to the developing brain. Corroborating the latter hypothesis, the BBB is starting to be established approximately at E13.5, at the time of fetal liver monocyte

release into the blood circulation (Daneman et al. 2010), but after YS-derived macrophages start to invade the neuro-epithelium from E9.0–E9.5 (Ginhoux et al. 2010; Ginhoux and Merad 2010), possibly restricting the access of fetal liver-derived cells to the embryonic brain (Fig. 9.2). This scenario likely parallels the observations in adult mice that inflammation and disruption of the BBB is required for monocytes to contribute to the microglial compartment (Fig. 9.3).

9.6 Microglial Contribution to CNS Development

Having discussed the generation and maintenance of the microglial compartment, we will now turn our attention to the function of this highly specialized cell population. The CNS is an immune-privileged site (Yang et al. 2010), which under physiological conditions is inaccessible to the majority of peripheral immune cells, soluble factors, and plasma proteins, due to the active protection of the BBB (Daneman 2012). However, the CNS still requires an effective system of immune surveillance and this is largely carried out by microglia which constantly scan the environment, monitor synapses (Wake et al. 2009), and clear apoptotic cells (Peri and Nusslein-Volhard 2008; Sierra et al. 2010). Upon detecting inflammation, infection, or cell death, microglia are activated to begin elimination of infectious agents and cell debris (Mallat et al. 2005; Perry et al. 2010; Ransohoff and Brown 2012; Ransohoff and Engelhardt 2012; Wynn et al. 2013), alongside production of neurotrophic and pro-inflammatory factors, chemokines, and cytokines (Kronfol and Remick 2000; Ueno et al. 2013). While these actions are required to neutralize a pathogenic threat to the CNS, the process of neuro-inflammation is also linked to the pathology of several neurodegenerative diseases (Chen et al. 2010; Perry et al. 2010; Prinz et al. 2011; Derecki et al. 2012; Kingwell 2012).

Alongside their immune roles, there is increasing evidence that microglia also dynamically interact with neuronal cells during brain development (Graeber 2010; Hughes 2012). Moreover, several neuropsychiatric disorders that are linked with defects in cerebral development have also been associated with microglial dysfunction (Vargas et al. 2005; Venneti et al. 2006; Pardo and Eberhart 2007; Brown 2011; Suzuki et al. 2013). In this section, we will give an overview of the current knowledge of microglia-CNS interactions (Fig. 9.4), focusing on their developmental functions and emerging role in the etiology of neuropsychiatric pathologies.

9.6.1 Microglial Colonization of the CNS

In mice, microglial cells can be detected in the neural folds of the brain rudiment starting from E9.0. Microglia gradually invade the cortical neuro-epithelium through the meninges, the lateral ventricles, and the choroid plexus (Verney et al. 2010; Swinnen et al. 2013) and, during all of embryonic development and for the first two postnatal weeks, proliferate in situ in the brain (Ginhoux et al. 2010), accounting for 1.7 % of total brain cells at E18, increasing to the 13 % at the end of

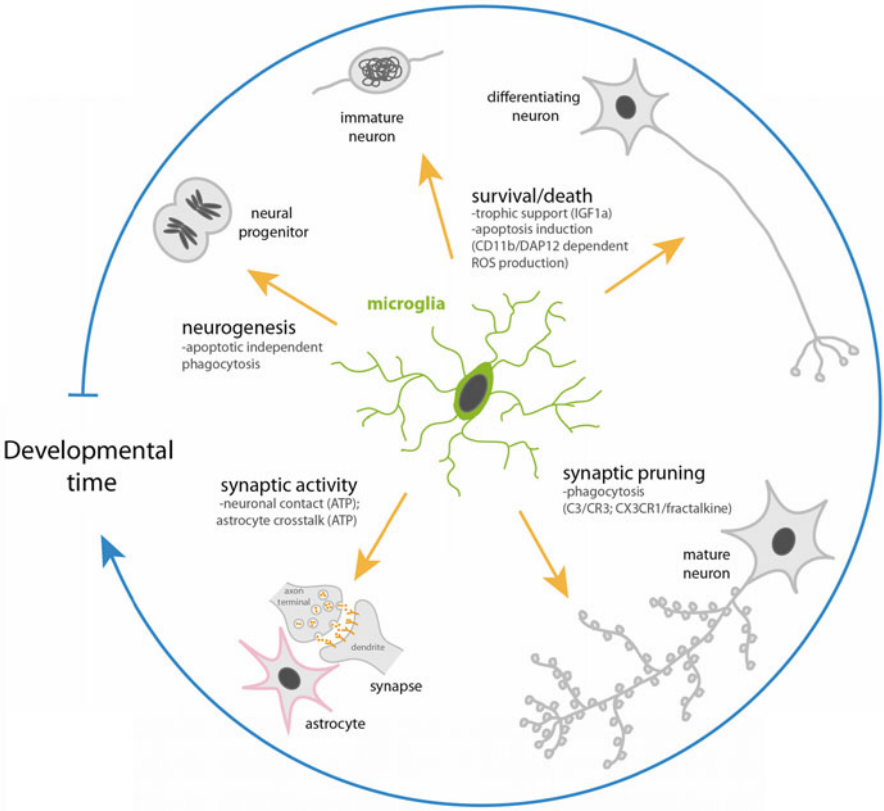

Fig. 9.4 Microglial functions in shaping the development of the CNS. Schematic representation of the processes regulated by microglia during the physiological development of the CNS at different developmental time points. Microglia (*green*) participate to neurogenesis, survival, and death of neuronal cells, trophic support for neurons, thereby regulating the number of progenitor and neuronal cells. They play a role also in the remodeling and maturation of synapses, through the involvement of different signaling pathways (C3/CR3 and CX3CR1/fractalkine) and they directly or indirectly (*via* astrocyte recruitment) modulate synaptic activity (*for detail*, *see text*) (color figure online)

the second postnatal week, when the blood brain barrier is already closed (Alliot et al. 1999). In adult, microglia account for about 5–20 % of adult CNS-cell population (Perry 1998) and appear in steady state highly ramified and homogeneously distributed (Fig. 9.1). In contrast, during embryogenesis and early postnatal development, these cells show ramified and amoeboid morphologies (Fig. 9.1) and display an uneven localization, forming specific high-cell-density hotspots in different brain regions, not necessarily in association with known sites of developmental programmed cell death (Ashwell 1991; Verney et al. 2010; Perry et al. 1985). While distinct morphologies of microglia are associated in the adult with a resting sentinel status or an activated status, the embryonic morphologies have yet to be associated with potentially specific functions.

9.6.2 Functions Within the Embryo

During neurogenesis of the cerebral cortex, an exuberant number of progenitors is generated in the proliferative ventricular and subventricular zones (VZ and SVZ), which is subsequently reduced during the terminal phases of cortical development (Bayer and Altman 1991). Recent data in both rodents and primates show that microglia contribute to this limiting process by regulating the cortical neuronal precursor pool through phagocytosis (Cunningham et al. 2013). In particular, during the later stages of rodent prenatal development (E17.5 onwards), microglia in the cortex were found to specifically phagocytose neuronal precursors expressing the transcription factor Tbr2 or Pax6. This phenomenon was not elicited by local apoptosis and was diminished after in utero microglial deactivation or elimination, and increased after maternal immune activation. In addition to this regulation of neurogenesis, our own studies (unpublished data) are revealing that microglia also modulate the progression of dopaminergic axons in the mouse forebrain, as well as the positioning of a population of migrating interneurons in the cerebral cortex. While the underlying cellular and molecular mechanisms remain to be characterized, it is now clear that microglia occupy a diverse repertoire of roles in the embryonic shaping of brain circuits. Since several neuropsychiatric diseases have been associated with abnormal embryonic brain development, these observations raised the possibility that defects in embryonic microglia might be implicated in CNS disease.

9.7 Microglial Roles in Shaping Postnatal Circuits

9.7.1 Regulation of Neurogenesis

The process of microglial phagocytosis of neuronal precursors and progenitors persists from embryonic development into, and throughout, adulthood (Dalmau et al. 2003; Sierra et al. 2010, 2013). Both amoeboid and ramified microglia have the ability to phagocytose apoptotic cells (Dalmau et al. 2003), and numerous phagosomes are evident in both cell types (Murabe and Sano 1982), implying that they are phagocytically active. However, the level of microglial phagocytosis seems to depend on their localization within the CNS, at least during the first postnatal week (Ashwell 1991; Sierra et al. 2013). Specifically, there is evidence that microglia are responsible for regulating the number of immature neurons born in the subventricular zone of the forebrain, one of the few sites of postnatal neurogenesis (Sierra et al. 2010, 2013).

9.7.2 Microglia Contribute to Death and Survival of Neuronal Cells

Beyond the elimination of apoptotic cells, microglia have recently been shown to actively and directly promote apoptosis in different parts of the CNS. In rodent cerebellum, the Purkinje Cell (PC) population, a main component of the cerebellar

efferent pathway, undergoes substantial apoptotic remodeling during the first postnatal week, which now seems to be driven by microglia. At postnatal day 3 (P3), microglial cells were described to clear apoptotic PC as expected, but interestingly, depletion of microglia in brain culture slices in vitro resulted in increased PC survival (van Rooijen et al. 1997). Another study revealed that PC apoptosis was being induced by superoxide ions generated from microglial respiratory bursts (Marin-Teva et al. 2004). Similarly, in perinatal mouse, reactive oxygen species produced by microglia enhance apoptosis of neurons in the hippocampus in vivo (Wakselman et al. 2008). Knocking out the CD11b and DAP12 genes, which are co-expressed specifically in developing microglia (Roumier et al. 2004; Block et al. 2007), the authors showed that microglial reactive oxygen species (ROS) production, with consequent hippocampal neuronal death, depends on CD11b/DAP12 integrin signaling (Wakselman et al. 2008), which is a known innate immune mechanism for induction of apoptosis.

In parallel to their negative regulation of neuronal cell numbers through induction of apoptosis, it now seems that microglia can promote neuronal survival. Postnatal inactivation or temporal elimination of microglia, using minocycline or the CD11b-DTR mouse, respectively, specifically increased the apoptosis of layer V cortical neurons. Postnatal microglial immune activation did not affect layer V cell death, thus apoptosis was indeed provoked by inactivation or absence of microglia, rather than by an inflammatory activation (Ueno et al. 2013). This might be explained by the observation that microglia produce substantial amounts of the trophic factor IGF1 in vitro, that binds the IGF1 receptor (IGF1Ra) expressed by layer V cortical neurons. Together, IGF1/IGF1Ra activate the PI3K–AKT pathway involved in cell survival. The importance of microglial IGF1 in vivo was subsequently confirmed by the use of IGF1R inhibitors and *igf1* siRNA, which both resulted in increased cell death of layer V cortical neurons (Ueno et al. 2013).

Collectively, these findings reveal the active participation of microglia in modulating neuronal death, either by inducing it or by promoting survival via provision of trophic support during early postnatal development.

9.7.3 Microglia Regulate Synaptic Pruning and Maturation

In addition to their diverse roles in modulating the size of the neuronal population, microglia are involved in the remodeling of neuronal connections during postnatal development. Cerebral functions rely on the formation of synaptic connections between neurons, and postnatal synaptic pruning leads to the elimination of synapses produced in excess during brain development. Recent studies have revealed the involvement of microglia in regulating this selective synaptic elimination, particularly in the retinogeniculate system (Kettenmann et al. 2013), which relays visual information from the retina to the dorsolateral geniculate nucleus of the thalamus, a major brain center. During postnatal development, visual inputs from the two eyes are segregated by microglial elimination of the weaker presynaptic connections formed earlier in development (Schafer et al. 2012). Not only does this demonstrate the functional importance of microglial phagocytosis in this system,

but also implies that microglia have the capacity to distinguish between stronger and weaker synapses (Schafer et al. 2012). Although the underlying mechanism has not yet been identified, the C3/C3 receptor (CR3) complement signaling pathway appears to be involved in microglial synapse recognition in this system. Indeed, mice deficient in C3 or CR3, classically expressed by neurons and immune cells respectively, exhibited defects in separation of visual inputs related to deficits in structural remodeling of synapses (Schafer et al. 2012). Synaptic refinement by microglia has also been reported in the murine hippocampus (Paolicelli et al. 2011). In particular, the interaction between microglial cells and synapses appears to be dependent on the CX3CR1/fractalkine signaling pathway, since *cx3cr1* knockout mice, in which microglial cells lack the receptor for fractalkine (Jung et al. 2000; Zhuang et al. 2007), exhibit features consistent with immature brain circuitry associated with transient reduced synaptic pruning. A transient lower density of microglia was also observed in the *cx3cr1* knockout mice, thus it is possible that this signaling pathway might promote microglial proliferation or migration into the brain. As a consequence of lower microglia density, the observed reduced synaptic pruning should be due to a deficit in microglial surveillance (Paolicelli et al. 2011). In addition, the CX3CR1/fractalkine signaling pathway has been shown also to regulate the maturation of thalamocortical synapses in the mouse somatosensory cortex (Hoshiko et al. 2012). Thalamocortical axons convey sensory information from the whisker map of the face to the neocortex. They invade the cortical layer IV around P4, forming barrel-like structures, in which fractalkine starts to be expressed, driving microglia entrance into the barrels. CX3CR1 inactivation in microglia not only delayed the recruitment of these cells into the barrels, but led also to a retarded functional maturation of thalamocortical synapses. In this context, no difference in global microglia density has been observed, rather it is the temporal delocalization of these cells or putative abnormal cell properties that influence the functional maturation of thalamocortical synapses. These findings highlight the importance of the precisely regulated spatio-temporal encounter between microglia and developing synapses, which is required to accomplish correct synaptic maturation. In addition, this also highlights the pertinent fact that modifications to microglial functions in crucial phases of development and refining periods could lead to long lasting neurological and physiological consequences.

9.7.4 Microglial Cells as Modulators of Neuronal Activity

In recent years, microglial cells have been shown to finely perceive changes in neuronal activity and, in response, to promptly act as homeostatic modulators in a variety of systems (Wake et al. 2009; Tremblay et al. 2010; Li et al. 2012; Pascual et al. 2012; Bechade et al. 2013). Using two-photon in vivo imaging of both the juvenile and adult rodent visual systems, it has been shown that interactions between highly motile microglial processes, axon terminals, and synaptic elements are extremely dynamic and respond to visual experience modulation (Wake et al. 2009; Tremblay et al. 2010).

Microglia seem also capable of reducing or initiating neuronal and synaptic activity (Li et al. 2012; Pascual et al. 2012). For example, in the optic tectum of zebrafish larvae, microglia constantly contact neuronal cell bodies in a non-random fashion, driven by release of ATP from highly activated neurons. Neurons are subsequently enclosed by microglial processes for up to a few minutes, which results in a lasting decrease in neuronal activity (Li et al. 2012). Furthermore, indirect activation of neuronal activity by microglia has been reported in hippocampal neurons in vitro following microglial activation. Microglia activated by lipopolysaccharide (LPS) release ATP, which then attracts astrocytes, which in turn liberate glutamate. Glutamate release leads to a fast and temporary increase in the frequency of spontaneous synaptic AMPAergic post-synaptic currents in hippocampal neurons (Pascual et al. 2012). These findings demonstrate how, in mature systems, microglia can contribute to modulation of neuronal and synaptic activity, and raise intriguing questions regarding their analogous functions in shaping CNS development.

9.8 Microglial Involvement in Developmental Pathologies

9.8.1 Microglia and Neuropsychiatric Diseases

Neuropsychiatric diseases including schizophrenia, autism spectrum disorders (ASD), and obsessive-compulsive disorder (OCD) are associated with defects in brain development, even though symptoms can appear in adolescence or early adulthood. Both ASD and schizophrenia involve a broad spectrum of symptomologies including disturbances in social interaction, communication, and the occurrence of repetitive stereotyped behaviors in ASD, and hallucinations, emotional deficits, and cognitive impairments in schizophrenia. Both disorders also have multifactorial etiologies that encompass genetic predisposition but perhaps include immunological factors as well (Pardo and Eberhart 2007; Brown 2011). Particularly, maternal inflammation during pregnancy, triggered either by bacterial or viral infections, constitutes a major risk factor for development of ASD and schizophrenia in the offspring (Ashwood et al. 2006; Patterson 2009). Furthermore, post-mortem immunocytochemical studies on the brains of autistic patients, coupled with Positron Emission Tomography imaging in living ASD-affected individuals, have revealed the presence of extensive neuroinflammatory processes with increased microglial activation in the white matter, cerebral cortex, and cerebellum (Vargas et al. 2005; Venneti et al. 2006; Suzuki et al. 2013). In addition, immune dysregulations, such as elevated levels of pro-inflammatory cytokines in serum, plasma, and cerebrospinal fluid have been reported in schizophrenic patients (Chew et al. 2013).

While such studies hinted at a correlative link with microglial activation, support for a causal role of microglia first came from analyses of rodent models exhibiting neuropsychiatric features. In the brain, the transcription factor hoxb8 is expressed exclusively by microglia; genetic inactivation of hoxb8 caused emergence of obsessive grooming behavior in mice, which mirrors the human OCD trichotillomania,

characterized by excessive body cleanliness and compulsive hair removal (Chen et al. 2010). Through a series of BM transplantation experiments, the authors also showed that wild type microglial-like cells generated from the donor BM were able to rescue excessive grooming behavior, demonstrating the direct link between microglial dysfunction and behavioral defects in this model. Whether these bone marrow-derived microglia can fully fulfill the functional roles of the endogenous embryonic microglia population remains to be investigated. Furthermore, impairments in cognitive functions have been reported in mice deficient for the microglia specific receptor for fractalkine, CX3CR1 (Jung et al. 2000). Beyond decreased hippocampal neurogenesis (Bachstetter et al. 2011), *cx3cr1*$^{-/-}$ and *cx3cr1*$^{+/-}$ mice showed motor learning deficits, deficiencies in associative and spatial memory, decreased Long Term Potentiation (LTP), and increased microglial activation (Rogers et al. 2011). Most remarkably, progression of behavioral symptoms in the mouse model for human Rett syndrome (an ASD) can be arrested by transplantation of wild type BM (Derecki et al. 2012). Further evidence of the impact of abnormal microglial activation in mice can be seen in both genetic (DAP12 knockout mice) and pharmacological animal models of maternal immune activation (poly I:C, LPS, influenza, bacterial infection), where common markers of schizophrenia are evident, such as typical behavioral and cognitive alterations and increased cytokine expression (Kaifu et al. 2003, Patterson 2009).

At this time, unequivocal data on the link between microglial alterations and the insurgence of neuropsychiatric diseases is lacking. However, considering the diverse physiological roles of microglia in brain development, dysfunction of these cells due to an inflammatory event could realistically result in defects of neurogenesis, neuronal survival, synaptic formation, and synaptic transmission. This in turn might begin to account for the role of prenatal inflammation in the occurrence of neuropsychiatric diseases, although when and how microglia act during pathological brain development remains to be determined.

9.8.2 Microglia in the Preterm Brain

Severe prematurity is a substantial risk factor for the development of several neuropsychiatric disorders, ranging from behavioral, cognitive, and emotional deficits, to cerebral palsy, autism, and schizophrenia (Verney et al. 2010; Johnson and Marlow 2011; Chew et al. 2013). Among the well known neurological consequences of severe preterm birth are altered brain development and focal brain injury, such as Periventricular White Matter Injury (PWMI) (Volpe 2001; Verney et al. 2010; Johnson and Marlow 2011). Multiple studies have reported increased microglial cell activation, accompanied by the production of several pro-inflammatory factors, in the white matter of preterm brains, specifically in the regions subjected to lesions (Monier et al. 2007; Chew et al. 2013). Although it is not clear from these observations whether, or how, microglial cells are involved in the occurrence of focal brain injuries, further investigations should provide more definitive evidences.

9.9 Concluding Remarks

Microglia, better known as the specialized immune cells of the CNS, are acquiring more and more relevance as active protagonists of CNS development (Fig. 9.4). While evidence accumulates for their lasting impact on circuit shaping, maturation, and refinement, a strong association between abnormal microglial activity during development and the occurrence of neuropsychiatric diseases is beginning to emerge. Nowadays, it is becoming increasingly clear that the origins of several, if not all, neuropsychiatric diseases can be dated back to developmental dysfunctions. However, it is also true that the CNS displays an incredible degree of plasticity, which allows, in several cases, for a prompt recovery of the defects during the first postnatal weeks after birth. Several diseases are indeed caused by multifactorial events, and they manifest only after reaching a certain threshold. In this context, microglial cells could be considered as "holding the balance of power," since the functions they exert point to the fine modulation and regulation of some phenomena, ultimately leading to brain remodeling and circuitry refinement. This balance of power could be changed by environmental factors that transiently modify microglia activity, such as prenatal inflammation.

Conversely, some neuropsychiatric diseases could be linked to genetic defects impairing microglial functions. In the later situation, the generation of in vitro derived microglial cells will constitute in the near future an essential experimental strategy. However, care needs to be taken on the source of microglial progenitors that should ideally closely mimic the endogenous development of microglia from YS primitive macrophages. In this regard, the use of embryonic stem cells (ESCs) or "induced pluripotent stem cell" (iPSC) to generate microglia is of great interest, as ESCs/iPSCs-derived hematopoiesis can recapitulate YS hematopoiesis in vitro (Ginhoux et al. 2013). Given their direct ontogenetic relationship, we postulate that techniques for directing primitive macrophage fate specification from pluripotent stem cells will also yield microglial precursors.

Overall, these recent discoveries are not only significant from a biological stand point, but moreover are prompting revolutionary changes in the way the scientific community considers the microglial compartment.

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Chapter 10
Kupffer Cells in Health and Disease

Andrea M. Woltman, Andre Boonstra, Makoto Naito, and Pieter J.M. Leenen

10.1 Introduction

The interstitial liver cells initially discovered by von Kupffer as "star cells" constitute the largest population of mononuclear phagocytes in the body. The strategic position of Kupffer cells (KC), at the luminal side of the liver sinusoidal endothelium, places them in an ideal position for their main function in the steady state, i.e. filtering of the blood that enters the liver from both the portal vein and the hepatic artery (see Fig. 10.1a, b for a schematic representation). In this manner they play an important role in the surveillance of potentially hazardous substances entering the body via the intestine, as well as in the recycling of iron by the removal of effete erythrocytes from the circulation. In addition to this scavenger function, KC are increasingly recognized as regulatory and effector cells in innate and adaptive immune responses to infectious agents and other conditions challenging liver homeostasis. Interestingly, KC activity also affects hepatocyte function directly, thus making these cells important versatile constituents of the liver.

In this chapter we will provide an overview of the phenotypic, developmental, and functional features of KC in the steady state as well as in disease. Given the

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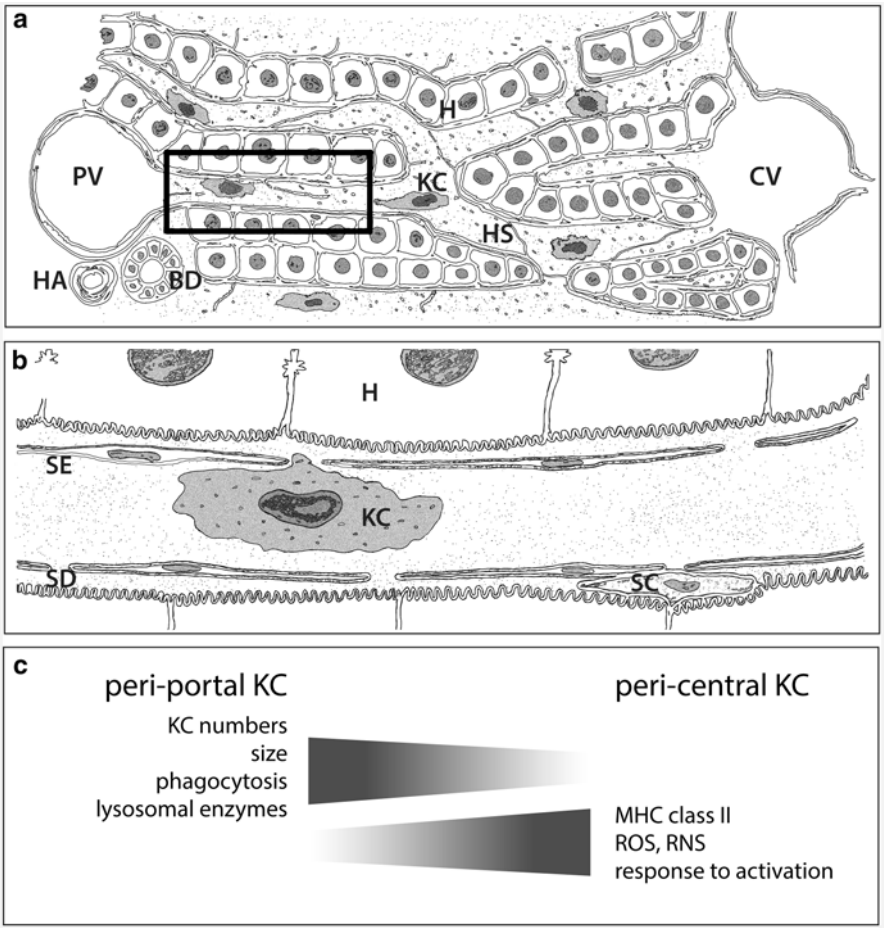

Fig. 10.1 Schematic representation of the liver micro-anatomical structure and Kupffer cell localization in lower (**a**) and higher magnification (**b**). (**a**). Hepatocytes (H) are arranged in plates, intermingled with hepatic sinusoids (HS) that transport blood from branches of the portal vein (PV) and hepatic artery (HA) to the central vein (CV). Together with the bile ducts (BD), portal veins and hepatic arteries constitute the portal area. Kupffer cells (KC) are found in the sinusoid lumen. (**b**) Between the hepatocytes and discontinuous, fenestrated sinus endothelial cells (SE), the space of Disse (SD) is located. Here, hepatic stellate cells (SC), also named Ito cells, are situated. Kupffer cells are, firmly or loosely, attached to the sinusoid wall. (**c**) KC in the portal areas differ in various aspects from those in the peri-central regions

ambition of this goal and the space limitations set, we necessarily restrict ourselves to the global picture as obtained from studies in human and in animal models, and refer to more specific literature where applicable. Although defining KC by their tissue localization and specific macrophage features seems clear-cut, distinguishing these cells from other myeloid cells such as circulating monocytes, perivascular macrophages, and different types of dendritic cells sometimes appears problematic. This especially concerns studying populations of cells after isolation and/or in

inflammatory conditions. In different studies, authors apply slightly different criteria for the identification of "genuine" KC vs. recent immigrants, and this easily creates confusion. Where possible, we attempt to clarify these semantic issues, but these are important considerations when interpreting experimental findings.

10.2 Kupffer Cell Phenotype

10.2.1 Tissue Localization and Morphology

Like various other tissue macrophages, KC have a characteristic amoeboid morphology with lamellipodia and an irregular surface containing many microvilli (Naito et al. 2004). They contain an ovoid or indented nucleus and their numerous cytoplasmic vesicles are reminiscent of a high level of phagocytic and pinocytic activity. Figure 10.2 provides an overview of histomorphological aspects of KC at different magnification levels. The cells adhere to the fenestrated sinusoidal

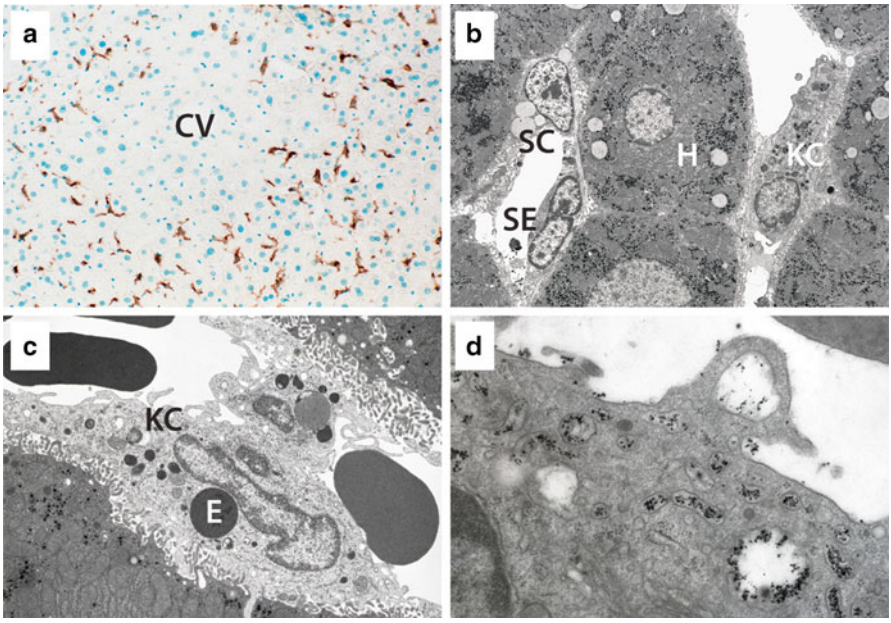

Fig. 10.2 Kupffer cell tissue localization and morphology at light microscopic (a) and electron microscopic level (b–d). (a) Mouse liver section stained with F4/80 antibody (brown) to show Kupffer cells. Nuclei are counterstained with methyl green pyronin. CV central vein. (b) Rat hepatocytes (H) and liver sinusoid with a Kupffer cell (KC), stellate cell (SC), and sinus endothelial cell (SE). (c) Rat KC with phagocytosed erythrocyte (E). (d) Surface of rat KC after 5 min of intravenous administration of thorotrast (radioactive thorium dioxide particles). Already after this short period of time particles are adhered to the cell surface and incorporated in (macro)pinocytic vesicles, and a worm-like structure

endothelium and are slightly more abundant in the peri-portal than in the peri-central regions. Although KC are the specialized phagocytes of the liver, sinusoidal endothelial cells of the liver have significant endocytic capacity, and this historically gave rise to the, now deserted, concept of the reticulo-endothelial system as scavenger system. In fact, early ultrastructural and functional studies were instrumental in establishing a definitive distinction between the two cell types (Wisse 1972). Both KC and endothelial cells contain high levels of lysosomal enzymes, such as glycosidases, nucleases, lipases, and proteases, in line with their clearance function. In mouse liver, KC numbers are approximately 35–40 % of the number of hepatocytes (Baratta et al. 2009), and comparable histological pictures in other species suggest very similar frequencies.

10.2.2 Kupffer Cell Molecular Phenotype

With regard to the KC's molecular phenotype, current approaches allow the acquisition of genome-wide information on expressed genes under steady state and experimental or diseased conditions. Ideally, this would provide elaborate phenotypic information for the comparison of KC in different circumstances, or with other cell types. However, the currently available information on KC gene expression profiles is relatively limited. In part this may be related to the demanding isolation procedures, which represent a challenge to achieve reliable gene expression profiles. In the recent comparison of different tissue macrophages by the Immunological Genome Consortium, KC proved elusive for definitive identification and/or isolation through sorting by flowcytometry (Gautier et al. 2012). At the time of writing of this chapter, the publicly accessible repositories (Geo and ArrayExpress) contain only two studies describing data on KC (Gorgani et al. 2008; Roudkenar et al. 2008). Other publications describe gene expression arrays of KC in various experimental settings, but the authors have not deposited raw data, thus limiting their value for subsequent use by others (Chen et al. 2010; Gehring et al. 2009; Xu et al. 2012; Zocco et al. 2006). In general, these studies strongly confirm the macrophage identity of KC. Comparison with other resident macrophages, however, also strengthens the profound influence of unique local conditions on the expression profiles and related biological functions. In this respect, KC are characterized by the high level expression of various types of receptors involved with endocytosis (Gorgani et al. 2008). Changing environmental conditions, for instance during liver regeneration or interferon treatment also have a significant impact on global gene expression by KC, showing their general responsiveness (Xu et al. 2012; Zocco et al. 2006).

To provide the reader with a general picture of the surface molecules characterizing KC, we limit ourselves to the general markers used for identification of the cells, and further emphasize on the receptors related to the main endocytic function of KC, i.e. complement and Fc receptors, scavenger receptors and C-type lectins (Table 10.1). This table summarizes data collected from published studies on

Table 10.1 Kupffer cell phenotype: an overview[a]

	Marker	Gene ID	Comment	Literature
Markers for KC identification				
Human	CD68		See below	
	CD14		See below	
Mouse	F4/80	Emr1	High level on resident KC	Hume et al. 1984. Anat Rec 210:503
	CD68		See below	
	CD11b		See below	
Rat	CD68/ED1		See below	
	CD163/ED2		See below	
Complement receptors				
	CRIg	VSIG4	Binds C3b and iC3b; required for phagocytosis of C′-coated pathogens; not described in rat	Helmy et al. 2006. Cell 124:915
	CR1/CD35	CR1	Uniform expression by human KC; only subsets in mouse; not described in rat	Hinglais et al. 1989. Lab Invest 61:509; Yan et al. 2000. Immunopharmacol 46:39
	CR3/CD11b	ITGAM	Low level on resident KC; high on recent immigrants	Hinglais et al. 1989. Lab Invest 61:509; Movita et al. (2012); Robinson et al. 1986. Immunology 57:239
	CR4/CD11c	ITGAX	Expressed by human KC; not by mouse KC; not described in rat	Hinglais et al. 1989. Lab Invest 61:509; Witmer-Pack et al. 1993. J. Cell. Sci. 105:965
Fc receptors[b]				
	Fc gamma RI/CD64	FCGR1A	Expressed by human and mouse KC; not described in rat	Tuijnman et al. 1993. APMIS 101:319; Otten et al. 2008. J Immunol 181:6829
	Fc gamma RIIb/CD32	FCGR2B	Expression increased in hepatitis	Tuijnman et al. 1993. APMIS 101:319; Ganesan et al. 2012. J Immunol 189:4981; Lovdal et al. 2001. Cell Biol Int 25:821
	Fc gamma RIII/CD16	FCGR3A/B	Expression increased in hepatitis	Bordessoule et al. 1993. Br J Haematol 83:370; Tomita et al. 1994. Hepatol 20:317; Lovdal et al. 2001. Cell Biol Int 25:821

(continued)

Table 10.1 (continued)

	Marker	Gene ID	Comment	Literature
	Fc gamma RIV	Fcgr4	Expressed by mouse KC; gene not identified in human	Otten et al. 2008. J Immunol 181:6829
	Fc alpha RI/CD89	FCAR	Expressed by human KC; gene identified in rat, not in mouse; increased in inflammation	van Egmond et al. (2000)
	Fc epsilon RII/CD23	FCER2	Subset of mouse KC positive (20–40 %)	Eicher et al. 1995. J Leukoc Biol 58:32
	Neonatal Fc receptor	FCRN	Expressed at high level by KC	Cianga et al. 2011. Hum Immunol 72:1176; Borvak et al. 1998. Int Immunol 10:1289
Scavenger receptors[c]				
Class A	SR-A/SCARA1/CD204	MSR1	Contributes to but not solely responsible for modified LDL clearance in liver; decreased expression upon LPS exposure in vivo	Tomokiyo et al. 2002. Atherosclerosis 161:123; Van Berkel et al. 1998. Biochem J 331 (Pt 1):29; Xie et al. 2002. Hepatobiliary Pancreat Dis Int 1:558
	MARCO/SCARA2	MARCO	Not expressed by KC in steady state; strongly induced in KC upon infection or LPS exposure; not described in rat	Elomaa et al. 1998. J Biol Chem 273:4530; van der Laan et al. 1999. J Immunol 162:939
Class B	SR-BI/CD36-like 1	SCARB1	R for HDL-cholesterol ester; high-fat diet up-regulates expression in KC, but down-regulates in hepatocytes	Nakagawa-Toyama et al. 2005. Atherosclerosis 183:75; Fluiter et al. 1998. J Biol Chem 273:8434
	SCARB3/CD36	CD36	Major R for modified LDL in rat and mouse KC; not detectable by IHC on human KC in steady state	De Rijke et al. 1994. Biochem J 304:69; Bordessoule et al. 1993. Br J Haematol 83:370; Bieghs et al. 2012. PLoS One 7:e34378
Class D	Macrosialin/CD68/ED1 (rat)	CD68	Generic marker for KC identification expressed by most KC in steady state and inflammation	Tomita et al. 1994. Hepatol 20:317; Movita et al. (2012); Gomes et al. 2004. Mol Aspects Med 25:183
Class G	SR-PSOX/CXCL16	CXCL16	Chemokine with dual function as scavenger R; strongly expressed by mouse KC; only occasional staining of human KC; not described for rat KC	Wehr et al. 2013. J Immunol 190:5226; Heydtmann et al. 2005. J Immunol 174:1055

Non-classified scavenger receptors				
	CD14	CD14	Only marginally expressed by KC in steady state; induced after LPS or ethanol in vivo	Tomita et al. 1994. Hepatol 20:317; Enomoto et al. 1998. Gastroenterology 115:443; Xie et al. 2002. Hepatobiliary Pancreat Dis Int 1:558
	CD163	CD163	Increasingly expressed by human KC in viral hepatitis; major subset of rat KC (ED2); uniform expression peri-portal, less peri-central	Hiraoka et al. 2005. Pathol Res Pract 201:379; Polfliet et al. 2006. Immunobiology 211:419; He et al. (2009)
C-type lectin receptors[d]				
	Mannose R/CD206	MRC1	Expressed in steady state; up-regulated by dexamethasone	Haltiwanger et al. 1986. J Biol Chem 261:15696; Zhu et al. 2004. J Pharmacol Exp Ther 308:705; Noorman et al. 1997. Hepatol 26:1303
	Dectin-1/beta-glucan R	CLEC7A	Expression reported in mouse KC; note that also CR3 binds beta-glucan	Reid et al. 2004. J Leukoc Biol 76:86; Thornton et al. 1996. J Immunol 156:1235
	Asialoglycoprotein R	ASGR1, -2	R for de-sialylated proteins	Coombs et al. 2006. Glycobiology 16:1C
	DC-SIGN/CLEC4L	CD209	Expression reported in human KC	Lai et al. 2006. Am J Pathol 169:200
	Kupffer cell R/CLECSF13	CLEC4F	Unique KC lectin in mouse and rat; R for Fuc and Gal, involved with glycolipid presentation to NKT cells; human ortholog is not expressed in liver and not a full-length protein	Linehan et al. 2000. Microbes Infect 2:279; Fadden et al. 2003. Glycobiology 13:529; Yang et al. 2013. PLoS One 8:e65070
	LSECtin	CLEC4G	Expression reported in human and mouse KC; negatively regulates activated T cells	Dominguez-Soto et al. 2009. Hepatol 49:287; Tang et al. 2009. Gastroenterology 137:1498-508 e1

[a] This table is restricted to markers used for KC identification and major surface receptors involved in endocytic function in human, mouse, and rat. Important species-specific or activation-related differences are indicated

[b] Recent reappraisal of Fc receptor diversity in different species (Bruhns 2012. Blood 119:5640) is not recognized in older literature

[c] For a recent overview of different scavenger R families in macrophages see Kzhyshkowska et al. 2012. Immunobiology 217:492

[d] See van den Berg et al. 2012. Ann N Y Acad Sci 1253:149 for a general overview

human, mouse, and rat KC. When applicable, species-specific differences are indicated. In addition to the indicated literature, readers are referred to reviews on KC (Huang et al. 2012; Crispe 2011; Naito et al. 2004).

10.2.3 Kupffer Cell Heterogeneity

Populating a large organ, with gradients of nutrients and oxygen from the portal to the central areas of the liver lobules, it is comprehensible that KC constitute a heterogeneous population of cells (Fig. 10.1c; discussed in Laskin et al. 2001; Naito et al. 2004). In general, KC in peri-portal regions are larger, more phagocytic, and contain higher levels of lysosomal enzyme activity, while being less responsive to inflammatory triggers. Conversely, the smaller KC in the peri-central areas express higher levels of MHC class II and produce more nitric oxide (NO) and superoxide anion upon stimulation. This diversity is also reflected in the differential expression of phenotypic and functional features of subpopulations isolated via counterflow centrifugation elutriation (e.g. ten Hagen et al. 1998).

The method of study has a significant impact on the observed heterogeneity among KC, which is caused by the differential representation of different subsets. In situ labeling of cells, mostly in tissue sections, obviously provides a picture of the full spectrum of KC. Upon isolation, however, Crispe and collaborators noted a striking discrepancy between the KC subsets represented in isolated populations characterized by flowcytometry, and those identified in situ (Klein et al. 2007). They identified a subpopulation of so-called "sessile" KC that was highly underrepresented among the cells isolated using enzymatic digestion. Since sessile KC are uniquely radio-resistant and have distinct developmental and functional features (see below), it is important to realize the impact of technical approach when interpreting experimental findings and comparing results from different studies.

Although characterization in situ is most reliable, identification of distinct subsets at this level is challenging due to the difficulty of quantification of marker expression. Kinoshita and collaborators identified by flowcytometry two major KC subsets in mouse liver, which both are F4/80$^+$ but differentially express CD68 and CD11b (Kinoshita et al. 2010). In our own recent histological analysis of mouse KC, we and others observed differential expression of CD68 and F4/80 by KC with only partial overlap (Movita et al. 2012; Lloyd et al. 2008). In addition, only a minority of F4/80$^+$ KC showed histologically detectable CD11b expression. Careful evaluation let us conclude that F4/80-low CD11b$^+$ cells in the liver resemble monocyte-related cells, and differ from tissue-resident KC, with the latter population being more difficult to isolate from the liver by perfusion or collagenase treatment (Movita et al. 2012; Kinoshita et al. 2010). This subset distinction was recently confirmed, showing that CD68$^+$ cells, but not CD11b$^+$ cells, expressed CRIg, MerTK, and CD64, and were involved with systemic bactericidal activities (Ikarashi et al. 2013). In contrast, CD11b$^+$ cells were pivotal in immunity against tumors. Clearly, scoring cells positive or negative in immunofluorescence or immunohistochemistry depends on mul-

tiple technical parameters, but the findings at this level illustrate the heterogeneity of KC in the steady state. Whether this represents the existence of distinct subsets or a spectrum of cells with different expression levels remains to be determined.

10.3 Kupffer Cell Origin and Growth Factor Dependence

The origin of KC in the adult steady state has long been debated, with different experimental approaches leading to different interpretations. These varied from the classic mononuclear phagocyte system view of an exclusive bone marrow (BM) origin, where circulating monocytes differentiate into KC, to an embryonic origin of the KC population, which is maintained via local proliferation. The proliferating cells could be either immature local precursor cells or mature KC themselves. Early studies, for instance, showed extensive mitosis of KC during the regeneration phase after partial hepatectomy (Widmann and Fahimi 1975). However, this could also represent proliferation of immigrants from the circulation. Functional depletion of BM cells by radioactive ^{89}Sr incorporation into bone strongly suggested the independent maintenance of KC (Yamada et al. 1990), but disparate opinions on KC origin existed for a long time in the scientific community.

Increasing light was shed on this matter by more recent BM transplantation experiments (Klein et al. 2007). In these, Crispe and colleagues identified in mice the radio-resistant "sessile" KC mentioned above. Four weeks after BM transplantation, approximately half of the KC population appeared to be of donor origin as determined at the histological level. In seeming contrast, flowcytometric analysis showed 99 % of F4/80-high, CD11b-low KC were donor-derived at this time point. This discrepancy could be explained by the presence of sessile KC, which are barely replaced and escape enzymatic isolation, and therefore flowcytometric identification. The sessile population was, however, completely depleted by in vivo treatment with liposome-encapsulated clodronate, and then replaced by donor-type cells. Thus, sessile KC could be either self-maintained or have acquired radioresistance and longevity, and still be hematogenous in origin. The experimental conditions, however, significantly challenge the resident KC population, and thus the obtained results leave room for diverse interpretations concerning the origin of KC in the steady state. In any case, the liposome-mediated depletion experiments gave no indication for the existence of an immature, non-phagocytic local precursor, which would have generated host-type KC. Rather, these experiments showed the potential of BM-derived cells to differentiate into genuine KC.

Recent lineage tracing studies provided more definitive answers on the KC origin in the steady state (Schulz et al. 2012; Yona et al. 2013). These findings have shown that KC, as well as other F4/80-high macrophage populations like brain microglia, derive from yolk sac macrophages, and are maintained in adult mice independently from BM hematopoietic stem cell-derived monocytes. Development of these F4/80-high macrophages does not depend on the transcription factor c-Myb, in contrast to the differentiation of adult blood monocytes and their progeny. The

F4/80-high/CD11b-low KC are embedded in the parenchyma, while BM-HSC-dependent macrophages with the reverse phenotype are mostly located around the larger vessels in the liver (Yona et al. 2013).

Together, the picture emerges that in steady state at least three populations of macrophages can be discerned in the mouse liver. The similarly sized sessile F4/80-high/CD11b-low KC and the enzymatically isolatable F4/80-high/CD11b-low KC both have a yolk sac origin and are self-maintained in the steady state. Of these subpopulations, the latter exclusively takes part in local inflammatory responses (Klein et al. 2007). Only upon serious challenge, when resident KC are affected, these will be replaced by BM-derived cells. In contrast, the third population of F4/80-low/CD11b-high cells is monocytic in origin, and may develop into perivascular liver tissue macrophages.

For their development KC depend strongly, but not absolutely, on CSF-1/M-CSF, as different studies observed a reduction of the KC population in CSF-1-deficient op/op mice to approximately 30–50 % of the cells compared to controls (summarized in Wiktor-Jedrzejczak and Gordon 1996). Activity of the alternative ligand for CSF-1 receptor, IL-34, probably explains the generation of these residual KC. Absence of only IL-34, in turn, does not affect KC development, in contrast to generation of epidermal Langerhans cells and microglia (Wang et al. 2012). The notion that CSF-1 is an important growth factor for KC is supported by the finding that KC fail to repopulate in adult CSF-1-deficient but IL-34-sufficient op/op mice after liposomal clodronate-mediated depletion, while KC reach normal numbers after 14 days in CSF-1-sufficient mice (Yamamoto et al. 2008). Exogenous CSF-1 supplementation restores KC development in op/op mice. Interestingly, it was shown recently that resident macrophage populations, including KC, are stimulated under type inflammatory type 2 conditions to proliferate in an IL-4-mediated, CSF-1-independent fashion (Jenkins et al. 2013).

KC not only depend on CSF-1 as growth factor, being the largest macrophage population in the body they are also important regulators of macrophage homeostasis in general. They do so, on the one hand, by functioning as a sink of circulating CSF-1 (Bartocci et al. 1987), and, on the other hand, by producing CSF-1 at significant levels upon demand (Moriyama et al. 1997).

10.4 Kupffer Cell Functions in Steady State

Under steady state conditions, in the absence of triggers by pathogens, disease or physical stress, KC fulfill an important role to eliminate insoluble macromolecules, immune complexes, toxins, and degenerated cells from the circulation. Since KC reside in the liver sinusoids in large numbers and are adherent to the endothelial cells they are able to sample the blood entering the liver from the gut as well as from the main circulation. Elimination of debris and insoluble macromolecules is via pattern recognition receptors (PRR), such as scavenger receptors, mannose receptors, and Fc receptors that are able to bind immune complexes or opsonized cells.

Senescent or damaged erythrocytes are removed in the liver by KC, but also by macrophages in spleen and bone marrow. Furthermore, during the lifespan of erythrocytes, part of their hemoglobin content as well as of their membrane is shed as vesicles. These hemoglobin-containing vesicles are rapidly removed from the circulation by liver KC and, to a lesser extent, macrophages from other tissues mainly by scavenger receptors (Willekens et al. 2005). Following phagocytosis and hemolysis, the different components are degraded and recycled. Hemoglobin is degraded by heme-oxygenases (HO), of which HO-1 is expressed in KC. HO-1 is induced by diverse stimuli including heme, heat stress, LPS, and various cytokines. HO-1 catalyzes the degradation of heme into iron, biliverdin, and carbon monoxide, which are all considered to be hepatoprotective at low quantities under steady state conditions. Iron is then either stored intracellularly as ferritin or conveyed to circulating transferrin via membrane-bound ferroportin. Interestingly, it was also shown that heme-degrading HO-1 acts as a downstream effector molecule of IL-10 that mediates its immunosuppressive activities (Lee and Chau 2002).

Removal of leukocytes like T cells or neutrophils from the circulation also takes place in the liver by KC. The importance of the liver in this respect is indicated by the finding that apoptotic neutrophils appeared in the lungs and spleen only after inactivation of KC by gadolinium chloride (Shi et al. 2001). Phagocytosis of apoptotic cells by KC is mediated by recognition of surface phosphatidylserine and, importantly, enhances the production of IL-10, and may favor a state of unresponsiveness. The induction of IL-10 may prevent development of sterile inflammation, activation of macrophages, and release of activating signals. Ongoing phagocytosis of apoptotic cells as part of liver homeostasis, in combination with low levels of LPS that are continuously released from the gut are likely responsible for constant low levels of anti-inflammatory mediators, such as IL-10, TGF-β and prostaglandins that restrict inflammation under steady state conditions. In addition, continuous exposure to serotonin probably contributes to the induction and maintenance of a non-inflammatory state (de las Casas-Engel et al. 2013).

10.5 Kupffer Cell Responsiveness

Macrophages are abundantly equipped with PRR to identify pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively). These PRR encompass multiple families, including Toll-, RIG-, and NOD-like receptors (TLR, RLR, NLR, respectively), and C-type lectins receptors (CLR). While macrophages in general express a range of these receptors, related to their sentinel function, not all of these have been described in KC. Mouse KC express TLR1–TLR9, all of which appear to be functional (Wu et al. 2009). Human KC so far have only been described to express TLR2, TLR3, TLR4 (Takii et al. 2005; Visvanathan et al. 2007). Furthermore, in the *Listeria monocytogenes* infection model, mouse KC are shown to express RIG-I (Imaizumi et al. 2006). Hepatocytes and $CD68^+$ liver mononuclear cells (presumably KC) express NLRC2 (NOD2) (Body-Malapel et al. 2008).

The ability of KC to produce various cytokines in significant amounts is still debated, mainly due to differences in KC definition and the used purification techniques (see discussion above). Previous studies on human and rat KC, using counterflow centrifugal elutriation, show that they are able to produce IL-10, TNF-α, and IL-6 upon LPS stimulation (Knolle et al. 1995; Kono et al. 2002). In addition, using flowcytometrically sorted cells and in the setting of bile duct ligation-induced liver inflammation, He et al demonstrated that $ED1^{+}ED2^{+}$ KC have a high mRNA level of IL-1β (He et al. 2009). In a mouse model of ischemia reperfusion, TLR9-mediated activation of the NLRP3 inflammasome by extracellular histones appeared to play an important role in IL-1 activation and liver damage (Huang et al. 2013). However, several other studies show that steady state KC are relatively weak in producing cytokines. In vitro stimulation with LPS, R848, or CpG resulted in very low levels of IL-10, TNF-α, and IL-12p40 produced by KC (Movita et al. 2012). Similarly, Kinoshita et al. (2010) showed that the $F4/80^{+}CD68^{+}$ KC produce only low levels of TNF-α and IL-12 upon in vitro LPS stimulation.

The reduced capacity of KC to produce pro-inflammatory cytokines has been related to their continuous exposure to bacterial products, such as LPS from Gram-negative bacteria in the gut, supplied via the portal vein (Knolle and Gerken 2000). This so-called endotoxin tolerance to repetitive antigenic stimulation prevents excessive activation of KC and inflammation of the liver. The state of unresponsiveness is mediated via immunosuppressive cytokines, such as IL-10 and TGF-β, and prostaglandins, and further supported by down-regulation of TLR expression levels and negative regulation of TLR signaling, for instance by IRAK-M (Liu et al. 2008). Active suppression by IL-10 and TGF-β reduces the antigen-presenting capacity of KC by down-regulating the expression of MHC molecules and co-stimulators, without strongly affecting the scavenger function of KC. The consequence of the immunosuppressive milieu on KC, but also on other potential antigen-presenting cells including dendritic cells and sinus endothelial cells, is allograft tolerance following liver transplantation, but also a limited ability to eliminate intrahepatic pathogens. It has been suggested that, depending on the microenvironment and the quality and strength of signals received by KC, they can become immune-stimulatory cells, as has been demonstrated in co-culture experiments with human NK cells (Tu et al. 2008).

10.6 Kupffer Cells in Disturbed Homeostasis

Under steady state conditions the distinction between tissue-resident KC and monocytes can be made on the basis of surface markers, but under inflammatory conditions this is more difficult due to changes in the expression of identifying markers such as F4/80, CD11b, and CD68, in the case of mouse KC (Beschin et al. 2013). In humans, this situation is even more complex. Studies on healthy KC generally use cells collected upon perfusion of donor livers prior to transplantation. These cells likely represent a selected subset as they are only loosely attached to the sinusoids. Phenotypic examination of these cells indicated that they are KC, although

contamination with peripheral monocytes cannot be excluded (Tu et al. 2008). Other studies on human KC use surplus liver material from diagnostic liver biopsies, which is mostly obtained from patients with chronic autoimmune diseases or chronic viral hepatitis to determine the severity of liver disease. However, in these chronic diseases the distinction between tissue-resident KC and inflammatory monocytes may be even more difficult, since the latter cells may have differentiated and established a long-lasting equilibrium during disease. Also, no distinctive surface markers are available, and the identification is generally performed using antibodies against CD14. In mice, the models used to study disturbed homeostasis are generally more acute, and chronic disease models have received less attention. Bearing these conceptual considerations in mind, we attempt to summarize in the next sections the contribution of KC to various diseases involving the liver, in particular metabolic disease, different types of infection, and liver injury.

10.6.1 Kupffer Cells in Metabolic Disease

KC have been implicated in various liver diseases with different etiologies that are associated with metabolic complications, such as over-nutrition, and may lead to fatty liver disease. Non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease in developed countries, and most patients carry the hallmarks of obesity and metabolic syndrome (Baffy 2009). NAFLD starts with steatosis, the accumulation of hepatic fat, which is generally followed by the increased production of ROS and secretion of pro-inflammatory mediators that may induce liver inflammation and injury. This inflammatory condition is recognized as non-alcoholic steatohepatitis (NASH).

The involvement of KC in NAFLD has long been recognized. In humans, increased numbers of CD68-expressing KC have been shown to correlate with histological severity of NAFLD (Park et al. 2007). In a mouse model of NAFLD, in which ob/ob mice are fed a high-fat diet, the total number of KC is not affected (Leroux et al. 2012). However, the function of KC is altered, since KC in NAFLD secrete higher levels of pro-inflammatory cytokines and express a so-called "M1-phenotype," which has also been shown for the increased numbers of macrophages present in obese adipose tissue (Lumeng et al. 2007). Enhanced secretion of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α by activated macrophages may lead to more tissue damage. The crucial role of KC in fatty liver disease has been indicated by experiments in mice where KC were eliminated by liposomal clodronate or by gadolinium chloride, which significantly decreased hepatic steatosis and insulin resistance (Huang et al. 2010; Rivera et al. 2007) and was accompanied by lower TNF-α and IL-6 mRNA expression.

The mechanisms driving KC activation in NAFLD and leading to hepatitis are beginning to be elucidated. Exposure of KC to elevated leptin levels, as present in metabolic syndrome, induce iNOS- and NADPH oxidase-mediated oxidative stress in KC, causing their activation (Chatterjee et al. 2013). Moreover, oxidized LDL

particles, but not unmodified or acetylated LDL, appear to accumulate in KC lysosomes and cause increased expression of inflammatory genes in the liver (Bieghs et al. 2013). Thus, the type of lipids to which KC are exposed play an important role in their polarization and activation, as was also indicated by a previous study by Papackova et al. (2012), in which high-fat diet rich in mono-unsaturated lipids stimulated an alternative M2-like program in KC, and not a classical M1 activation program characterized by pro-inflammatory cytokine production.

Recently, it has been shown that TNF-α-producing KC are crucial during the early stages of NAFLD to promote blood monocyte infiltration of the liver. Importantly, targeted knockdown of TNF-α expression by siRNA in myeloid cells decreases the incidence of NAFLD development by decreasing steatosis, liver damage, monocyte infiltration, and the production of inflammatory chemokines (Tosello-Trampont et al. 2012). In line with the observation that enhanced and altered activation of KC promotes pathogenesis of NAFLD, the well-known suppressive effects of IL-10 and IL-1 receptor antagonist ameliorate steatohepatitis in mice (Byun et al. 2013; Petrasek et al. 2012). Interestingly, it was recently shown that IL-10 released by M2-like alternatively activated KC stimulated apoptotic death of pro-inflammatory M1-like cells (Wan et al. 2014). This mechanism mediated resistance to hepatocyte steatosis and subsequent death.

Triggering of TLR4 by gut-derived LPS, which leads to activation of KC, is considered a key event in the pathogenesis of NAFLD (Baffy 2009). Remarkably, administration of LPS exacerbates liver injury in a model for NAFLD, whereas peptidoglycan administration, triggering TLR2/6, does not. One of the initiation events of NAFLD is likely an alteration in the intestinal microbiota due to overnutrition or metabolic disturbances. These changes in intestinal microbial profiles affect fat accumulation in the liver. In this respect, it has been shown that ob/ob mice, which are a well-known model for obesity, display a different microbial profile compared to control mice (Tilg 2010). As a consequence of altered gut microbiota the local as well as systemic levels of endotoxins, such as LPS, may increase and affect innate immune cells, including KC. Furthermore, and this is therapeutically extremely interesting, probiotics have been shown to have a beneficial effect on NAFLD in mouse models by reducing hepatic injury and inflammation, which further supports the crucial role of gut-derived LPS in initiating and maintaining NAFLD (Farrell et al. 2012).

Besides gut microbiota also other parameters involving KC influence NAFLD. Activity of distinct PPAR members have an effect on NAFLD pathogenesis, since PPAR-γ signaling improves insulin sensitivity and may be beneficial for NAFLD treatment, whereas lower levels of PPAR-α and PPAR-δ lead to increased fat storage and consequently more severe disease (Stienstra et al. 2010). In this respect, macrophage-specific induction of PPAR-δ signaling stimulates alternative or M2-like activation of KC, which is thought to mediate insulin-sensitizing effects (Odegaard et al. 2008). In addition, mediators produced by adipose tissue, such as leptins and other cytokines, may modulate KC function. In this regard, adiponectin may inhibit NK-κB and Erk1/2 signaling and thereby inhibit cytokine induction, whereas leptins induce the expression of pro-inflammatory cytokines.

Similar as in NAFLD, fatty liver disease caused by excessive alcohol consumption also leads to higher endotoxin levels in serum, which is primarily due to changes in the microbial gut flora and enhanced intestinal permeability (Thurman et al. 1998). Although the initiating trigger that causes alcoholic liver disease is different from NAFLD, many of the clinical observations and their underlying mechanisms are similar. Ethanol may directly affect the transcriptional regulation of genes involved in lipid metabolism, such as PPAR-α. Similar as in NAFLD, depletion of KC in mice also attenuates alcohol-induced disease, demonstrating a central role for KC (Nath and Szabo 2009). In addition, abrogation of the TNF-α pathway in vivo using antibodies against TNF-α or TNF-αRI gene-targeted mice strongly reduces alcohol-induced liver pathology, as demonstrated by reduced serum ALT levels and pathology scores (Iimuro et al. 1997; Yin et al. 1999). Interestingly, signaling via TLR4 activates KC in these mouse models, with the TRIF pathway—but not the MyD88 pathway—being responsible for TNF-α induction (Hritz et al. 2008).

10.6.2 Kupffer Cells in Infection

KC form, together with the sinusoidal endothelial cells, the first barrier for pathogens to enter the liver via the portal vein (Vollmar and Menger 2009). Their endocytic capacity, expression of different PRR, MHC and co-stimulatory molecules, and ability to produce cytokines upon stimulation, renders them potent immune cells contributing to either pathogen clearance or persistence. Pathogen recognition may activate KC leading to the production of pro-inflammatory mediators important for inhibition of pathogen replication, the induction of resistance to infection of neighboring cells, and attraction and activation of other immune cells. KC are the prime cells in the liver to present lipid antigens in a CD1-restricted manner to NKT cells. In conjunction, KC activate NK cells (also called Pit cells, representing the large intrahepatic NK cell population) and NKT cells via the production of pro-inflammatory cytokines, which on their turn produce activating cytokines such as IFN-γ and provide cytotoxic activity (Tu et al. 2008; Dao et al. 1998). Although liver macrophages are probably not involved in priming of naïve T cells, mouse KC have been shown to present antigen to $CD4^+$ and $CD8^+$ T cells, inducing these to proliferate and produce IFN-γ (Ebrahimkhani et al. 2011; You et al. 2008). The interaction of KC with membrane-bound as well as soluble mediators expressed by infiltrating immune cells probably leads to further regulation of KC function and of the intrahepatic inflammatory response. In contrast to their role as effective host defense cells favoring the survival of the infected host, evidence is accumulating that some pathogens use the transport properties of sinusoidal cells, including KC, to increase the efficiency of hepatocyte infection. Furthermore, pathogens may exploit the tolerogenic capacities of KC to evade immunity and/or may have evolved to inhibit the immunogenic functions of KC. In the following paragraphs we will provide examples of the various roles of KC in viral, bacterial, and parasitic infection, without the ambition, however, to be elaborate in the discussion on the different pathogens that challenge hepatic homeostasis.

10.6.2.1 Liver Infection by Viruses: Mouse Models

The defensive role of KC, as the first line barrier, to take up viral particles and thereby limit infection, has been shown in lymphocytic choriomeningitis virus (LCMV), murine hepatitis virus (MHV), and adenovirus infection (Lang et al. 2010; Pereira et al. 1984; Smith et al. 2008). Failure in direct clearing of viruses resulted in "spill-over" infection to hepatocytes, which prolonged the duration of infection and exacerbated immunopathology. Furthermore, the number of intrahepatic F4/80$^+$ cells increased during LCMV infection, suggesting the involvement of KC and/or recruited inflammatory monocytes in the immune response to LCMV (Dixon et al. 1986) and in the regulation of immunopathology (Lang et al. 2010). In mouse cytomegalovirus (MCMV) and adenovirus infection, KC were found to produce pro-inflammatory cytokines and chemokines, which were directly or indirectly responsible for monocyte, NK cell, and T cell infiltration in the liver (Salazar-Mather and Hokeness 2006; Lieber et al. 1997; Liu et al. 2003; Zaiss et al. 2002). Instead of promoting the expression of pro-inflammatory signals, MHV infection was found to down-regulate the production of IL-10 and PGE_2 by mouse KC (Jacques et al. 2008).

In general, the mouse models of viral hepatitis have generated much insight in disease pathogenesis, but fall short in important aspects, unfortunately. In contrast to the most common human hepatitis viruses, i.e., hepatitis A, B, and C viruses (HAV, HBV, and HCV, respectively) the mouse viruses also infect cells other than hepatocytes, including KC, and even other organs. Due to the narrow host specificity, studies on the anti-viral immune functions of KC in human hepatitis viruses are limited.

10.6.2.2 Liver Infection by Viruses: Human Hepatotropic Viruses

Both HBV and HCV are blood-borne viruses that can cause chronic liver disease and may elicit progressive liver injury leading to increased risk of developing liver cirrhosis, liver failure, and liver cancer. A very low number of HBV particles (< 10) is sufficient to establish hepatocyte infection in vivo (Jilbert et al. 1996; Asabe et al. 2009), indicating that liver targeting by HBV is extremely efficient. This may be enabled by initial scavenging of the virus by endothelial cells, as described for duck HBV (Breiner et al. 2001), or by other sinusoidal cells.

Productive infection of KC by HBV has been suggested on the basis of viral HBsAg-positivity of KC using suboptimal detection methods (Deodhar et al. 1975), but detailed information on the presence of HBV (proteins) in KC in vivo or the uptake of HBV or its proteins by human KC ex vivo has not been reported. So far, it is mostly unclear which receptors KC may use to recognize and take up HBV. Possible candidate receptors for direct binding of HBV to KC are heparan sulfate proteoglycan (HSPG), CD14, and the mannose receptor. Another molecule that binds HBsAg avidly is albumin, which is efficiently taken up by KC as well as hepatocytes, and may offer the virus a physiological transport pathway to parenchymal cells (Wright et al. 1988). Similarly, KC also express several receptors known

to be involved in hepatocyte infection by HCV (Ploss and Evans 2012), including HSPG (Pradel et al. 2002), LDL-receptor (Kleinherenbrink-Stins et al. 1991; Kamps et al. 1991), SR-B1 (Terpstra and van Berkel 2000), CD81 (Petracca et al. 2000), and DC-SIGN (Tu et al. 2008), a lectin known to bind the HCV envelope protein E2 (Pohlmann et al. 2003). Liver biopsy-derived cells exposed to E2 demonstrated binding to liver cells including KC (Petracca et al. 2000), but so far, there is no evidence for the occurrence of HCV *trans*-infection in vivo.

Exposure of KC to HCV-derived proteins leads to activation and the expression of pro-inflammatory factors, including IL-1β and TNF-α (Tu et al. 2010). Similarly, stimulation of human non-parenchymal cells, presumably KC, with HBV leads to the activation of NF-κB and production of IL-1β, IL-6, and TNF-α, cytokines known to inhibit HBV replication in hepatocytes (Hosel et al. 2009). However, when incubating rat KC with HBV virions, they hardly expressed IL-1β, IL-6, or TNF-α, but instead preferably produced TGF-β (Li et al. 2012). Likewise, also HCV has been shown to induce the production of immune regulatory factors including IL-10 by monocyte-derived macrophages, which may promote viral persistence (Chang et al. 2007). A recent study, investigating the difference between responders and non-responders to IFN-α therapy against HCV, showed that non-responders had relatively high expression levels before treatment of IFN-stimulated genes in the liver (Lau et al. 2013). In these patients, KC appeared to be a local source of type 1 IFN that promoted basal expression of IFN-stimulated genes in hepatocytes, and thus negatively influenced the outcome of IFN-based therapy.

The PRR, either extracellularly or intracellularly expressed, needed for these responses to viral proteins remain to be identified. TLR2 has been put forward as a possible specific recognition receptor for the nucleocapsid protein of HBV, i.e. HBcAg, and the non-particulate version of this protein, HBeAg. Moreover, HBeAg present in the cytosol seems to interfere actively with TLR signaling (Lang et al. 2011). TLR2 has also been suggested to play a role in HCV-core and nonstructural-3 protein (NS3) recognition by macrophages leading to MyD88-dependent activation, including increased IRAK activity, p38 phosphorylation, ERK and JNK activity, and AP-1 activation (Imran et al. 2012). Furthermore, it was shown that NS3-induced TNF-α production by KC is partially TLR4-dependent (Hosomura et al. 2011).

Due to high-level expression of adhesion molecules, KC can trap activated T cells and other cells in the liver sinusoids (John and Crispe 2004) leading to either immune activation or tolerance, depending on the status of the KC. It was reported that KC from chronic hepatitis C patients display an activated phenotype and form clusters with T lymphocytes, suggesting an interaction between KC and intrahepatic T cells (Burgio et al. 1998). However, KC derived from hepatitis B or C patients with chronic disease or KC exposed to these viruses in vitro were found to express also enhanced levels of immune regulatory molecules including PD-L1 and galectin-9, the ligands for PD-1 and TIM3 on T cells, respectively, which may suppress the activity of intrahepatic virus-specific T cells (Nebbia et al. 2012; Mengshol et al. 2010; Tu et al. 2010).

In contrast to HBV and HCV, HAV infection is self-limiting and does not induce chronic infectious disease. Although in vivo evidence is lacking, it has been

proposed that HAV reaches hepatocytes via KC that bind complexes of HAV and HAV-specific IgA antibodies via the Fcα receptor (van Egmond et al. 2000), and subsequently transfer the virus to hepatocytes. In contrast to HBV and HCV, HAV requires the disruption of host cell membranes to release its progeny. These dying hepatocytes may provide DAMP, such as ATP and nucleic acids, which can be recognized by KC and other intrahepatic immune cells, leading to activation of these cells that can overcome viral immune escape and liver-intrinsic tolerogenic mechanisms (Canbay et al. 2003).

Recently a direct contribution has been reported of KC to the pathogenesis of hepatitis in response to infection with viruses with tropism for other organs and not detected in the liver (Polakos et al. 2006). In influenza infection, KC were indicated as the effector cells killing hepatocytes in an as yet unidentified manner, leading to damage-associated hepatitis. KC can kill hepatocytes either directly via CD95-dependent apoptotic pathways or indirectly by interacting with $CD8^+$ (and possibly $CD4^+$) lymphocytes through stimulation of cytokine secretion and other mediators like phospholipases and NO (Polakos et al. 2006; Kolios et al. 2006). Although such a mechanism might explain the hepatitis observed in influenza, measles, SARS, and CMV infection, where the virus is not identified in the liver, a similar mechanism could well operate in the pathogenesis of hepatitis induced by hepatotropic viruses like HBV and HCV.

10.6.2.3 Liver Infection by Bacteria

Exclusive infection of the liver by bacteria has not been described, and most bacteria that reach the liver through the blood are efficiently cleared by immune cells. A major role for KC in host defense against bacterial infection is indicated by several studies in experimental models. Infection of mice with *Listeria monocytogenes* is a well-studied liver infection model. On the one hand, *Listeria* infection is dependent on KC function, as accumulation of bacteria in the liver depends on recognition of bacterial surface molecules by cognate receptors on KC (Ofek and Sharon 1988). On the other hand, production of inflammatory mediators such as IL-6, IL-12, IL-1β, TNF-α, and NO by infected KC inhibits proliferation of the microorganism (Ehlers et al. 1992; Ofek and Sharon 1988). At the same time KC-derived chemokines such as MIP-1α (CCL3), MIP-1β (CCL4), MCP-1 (CCL2), and MIP-2 (CXCL2 /-3), drive monocyte and neutrophil recruitment into the liver in order to control infection (Salkowski et al. 1998; Barsig et al. 1998; Ebe et al. 1999). In line with this, LPS treatment prior to infection has been shown to increase KC numbers leading to a reduction of bacterial load and improvement of prognosis in a *Salmonella* septicemia model (Lehner et al. 2001). Thus, as expected, KC inactivation or depletion results in impaired bacterial clearance (Cousens and Wing 2000; Tomioka et al. 2000; Pinto et al. 1991). In humans, the increased frequency of septicemia and septic shock involving Gram-negative bacteria in patients with hepatic failure have been attributed to the inability of KC to clear the portal circulation of microorganisms and endotoxin (Wyke 1987; Triger and Wright 1973).

Although KC play a critical role in blood clearance, various studies indicate that the actual elimination of the bulk of bacteria taken up by the liver depends on a complex interaction of KC and neutrophils that immigrate rapidly into the liver in response to infection (Gregory et al. 2002; Shi et al. 1996, 2001). While neutrophils are bactericidal cells par excellence, KC have a limited capacity to kill *Listeria*, for instance (Tomioka et al. 2000). Several mouse and rat studies demonstrated the presence of apoptotic neutrophils in KC upon bacterial infection or endotoxin exposure (Shi et al. 1996, 2001; Gregory et al. 2002). These findings suggest that KC play a critical role in eliminating neutrophils that accumulate in the liver sinusoids subsequent to clearance of bacteria, bacterial endotoxin, and microbial debris from the blood. Furthermore, it was found that neutrophils, accumulated in the liver sinusoids, suppress cytokine and chemokine production by KC, thereby showing an important role for neutrophil-KC interaction in moderating the pro-inflammatory response to bacteria taken up by the liver (Holub et al. 2009).

10.6.2.4 Liver Infection by Parasites

KC also represent the port of liver entry for parasites such as *Plasmodium* and *Leishmania*, which parasitize KC and then infect other liver cells (Tavares et al. 2013). Following the delivery of malaria parasites into the skin by a mosquito bite, the rapid migration of sporozoites allows them to escape clearance by local tissue phagocytic cells and to enter lymphatics and blood vessels. Via the blood, sporozoites rapidly reach the liver and, after gliding on HSPG in liver sinusoids, they use circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) to bind to KC (Pradel and Frevert 2001). Interaction with and passage through KC is important for hepatocyte infection (Ishino et al. 2004; Baer et al. 2007), indicating that these parasites use KC to overcome the sinusoidal barrier and, ultimately, to infect hepatocytes (Mota et al. 2002). TREM2 expression by KC appears to be an important determinant in resistance to liver stage infection against *Plasmodium* parasites (Goncalves et al. 2013). Once inside a hepatocyte, the parasites develop into merozoites, which will be released from the liver to infect erythrocytes (Sturm et al. 2006). Taken together, these data show that sporozoites not only use their migratory capacity to escape elimination by phagocytic cells, but also use KC to increase their efficiency at infecting hepatocytes.

10.6.3 Kupffer Cells in Liver Injury

Liver inflammation is initiated in response to a variety of signals to protect hepatocytes against damage and to favor tissue repair. KC, as prime sentinel cells, play an important role in initiating inflammation, while both KC and inflammatory macrophages contribute significantly to repair once the triggering factor has been eliminated (You et al. 2013). However, persistent or too intense inflammatory responses

will induce massive death of hepatocytes and hence cause irreversible tissue damage. Chronic inflammation is the basis of liver fibrosis and cirrhosis and also significantly increases the risk for hepatocellular carcinoma. Since KC, as major sentinel cells, contribute significantly to inflammation and its sequels, they play an important role in the induction and regulation of liver fibrosis and hepatocellular carcinoma. The knowledge on the role of KC in these processes is mainly obtained from experimental models in rodents.

10.6.3.1 Kupffer Cells and Liver Fibrosis

Liver fibrosis occurs as a wound-healing scar response following chronic liver inflammation and is characterized by excess collagen deposition and accumulation of extracellular matrix in response to chronic hepatocellular damage. Macrophage numbers increase in damaged liver and they are principally located around the regions of damage and fibrosis (Wallace et al. 2008). Inhibition of KC function using gadolinium chloride was found to reduce liver fibrosis in experimental models (Rivera et al. 2001). KC are thought to be involved in fibrogenesis via the production of ROS, cytokines, and growth factors that induce hepatic stellate cell myofibroblastic transformation (Wallace et al. 2008). In addition, they not only produce metalloproteinases and their inhibitors but also regulate the production of these factors by other cells, and stimulate their survival, leading to disturbance of the homeostatic mechanisms involved in extracellular matrix deposition (Xidakis et al. 2005; Pradere et al. 2013). Recent studies demonstrate that these actions are only partially conducted by liver-resident macrophages, but largely depend on recruitment of Ly6C-expressing monocytes as precursors of tissue macrophages into the inflamed and damaged liver (Imamura et al. 2005; Karlmark et al. 2009).

TGF-β is considered the main cytokine that drives fibrosis upon hepatic damage (Czaja et al. 1989; Castilla et al. 1991). KC-derived TGF-β has been suggested to drive hepatic stellate cell transformation and to induce production of collagen and proteoglycans by these cells (Meyer et al. 1990). In this context, IL-17A, which has been found elevated in fibrosis associated with alcoholic liver disease, appears to stimulate both KC and stellate cell activation (Hara et al. 2013). In vitro studies have shown that KC also can induce expression of platelet-derived growth factor (PDGF) receptors on hepatic stellate cells, thus enhancing stellate cell proliferation in response to PDGF produced by activated macrophages and stellate cells (Friedman and Arthur 1989). TNF-α, IL-1, and MCP-1 (CCL2), that are produced by activated KC, are also mitogenic and chemoattractant for hepatic stellate cells (Marra et al. 1999; Matsuoka et al. 1989). In addition, TGF-β and IL-6 induce mRNA expression of metalloproteinases and also their specific inhibitors in hepatocytes, KC, and hepatic stellate cells in rat liver (Knittel et al. 1999).

Experimental animal models indicate that monocytes/macrophages are not only critical for fibrosis progression, but also for fibrosis regression (reviewed in Wynn and Barron 2010). In the CCl_4 model of liver fibrosis, Duffield et al. (2005) demonstrated

that macrophage depletion during the fibrosis resolution phase impeded matrix degradation. An anti-fibrotic effect of liver macrophages was also demonstrated when macrophage infiltration was blocked during the induction of fibrogenesis in rats (Imamura et al. 2005). With regard to recovery from fibrosis, macrophages secrete proteinases that promote the degradation of scarring extracellular matrix proteins.

10.6.3.2 Kupffer Cells in Hepatocellular Carcinoma and Liver Metastases

Chronic hepatitis and cirrhosis are major risk factors for the development of hepatocellular carcinoma. In these conditions, hepatocytes are killed, and KC as well as other cells are activated to produce cytokines, such as hepatocyte growth factor (HGF), IL-6, and TNF-α, that drive the compensatory proliferation of surviving hepatocytes (Maeda et al. 2005). Dying hepatocytes trigger the inflammatory response by activation of KC. Recently, it was demonstrated that the expression of TREM-1 by mouse KC plays a crucial role in their activation upon recognition of necrotic hepatocytes (Wu et al. 2012). The augmented proliferation rate of hepatocytes increases the probability of genomic mutation in these cells. In addition, there are several possible mechanisms by which inflammation and inflammatory mediators may lead to genetic alterations.

Besides primary liver cancer, liver metastases are frequently observed, especially in gastro-intestinal malignancies. Hepatic metastases result from initial detachment of tumor cells from the primary site, entry into the portal circulation, and entrapment of metastatic cells in the liver sinusoids (Van den Eynden et al. 2013). In vivo microscopy has shown that KC are attracted to tumor cells in the hepatic circulation and have the ability to phagocytose these cells (Kan et al. 1995). NO, produced by KC after stimulation with TNF-α, PGE_2 (Valatas et al. 2004; Gaillard et al. 1991), and endotoxin derived from the portal circulation, is thought to be an effective weapon of the KC machinery against tumor cells, since NO is able to inhibit proliferation and induce apoptosis in cancer cells (Aono et al. 1994; Hussain and Harris 2007). However, NO also mediates tumor-promoting effects that include the ability to induce DNA damage, to increase angiogenesis by inducing VEGF production, to stimulate tumor cell proliferation and invasion, and to suppress anti-tumor immunity (Hussain and Harris 2007; Koblish et al. 1998). Furthermore, KC are able to produce HGF, which has been shown to contribute to tumor cell proliferation, and KC might facilitate tumor angiogenesis and invasion by secreting proteases, which alter the extracellular matrix in favor of tumor progression (Knittel et al. 1999). Moreover, KC may contribute to an immunosuppressive microenvironment by expressing immune regulatory factors, such as IL-10, PD-L1 in addition to NO, that could prevent an effective immune response towards the tumor (Trinchieri 2012).

Although KC may have ambivalent roles in interaction with tumor cells, the protective role of KC against hepatic metastases has been emphasized by the finding that KC depletion prior to tumor cell challenge resulted in a drastic increase in tumor development in the liver (Paschos et al. 2010; Kruse et al. 2013). In support

of this protective role of KC, isolated KC were found to be cytotoxic against human colon adenocarcinoma cells and this cytotoxicity was increased significantly when KC were stimulated with IFN-γ and endotoxin (Roh et al. 1990; Heuff et al. 1995). Other studies have demonstrated that KC induce Fas expression in colon cancer cells (Song et al. 2001) and malignant glioma cells (Lau et al. 2001) leading to Fas-mediated apoptosis and death in the presence of tumor-infiltrating lymphocytes or TNF-α. The opposing tumor-restricting and -promoting role of KC was elegantly modeled recently by depleting KC at different time points during tumor induction (Wen et al. 2013). Depletion prior to tumor transfer facilitated tumor growth, while late stage KC depletion led to decreased tumor mass.

10.7 Concluding Remarks

Studies in experimental animal models in particular have indicated that KC and liver-infiltrating macrophages contribute significantly to homeostasis in health and disease. They perform important tasks in protecting the host against invading pathogens and are thought to play a major role in induction and maintenance of immunological tolerance. Furthermore, evidence is accumulating that liver macrophages are essential for the resolution of fibrosis and display anti-tumor activities. However, they are also at least partly responsible for the development of liver diseases, including fatty liver disease, the initiation of various forms of hepatitis and sequellae, like fibrosis. Several microbial pathogens seem to use KC as transport cells to infect the liver. Once liver infection is established and/or liver cell damage has occurred, liver macrophages contribute importantly to the inflammatory response and further tissue damage by producing pro-inflammatory and pro-fibrotic mediators, by killing hepatocytes and even by supporting carcinogenesis.

The exact contributions of liver-resident KC vs. liver-infiltrating monocyte-derived macrophages to various processes of homeostasis and disease pathogenesis are difficult to determine, because of the highly overlapping characteristics of these cells. Moreover, the notion that infiltrating monocytes readily develop into KC in non-steady state conditions seemingly makes the distinction between “true” KC and recent immigrants also a semantic issue. However, resident KC and liver-infiltrating macrophages probably respond differently to various environmental conditions, given their distinct developmental histories, thus leading to diverse phenotypic and functional states of these cells. It is tempting to speculate that these differences may eventually be essential in the contribution of different liver macrophages to various homeostatic processes. With our growing appreciation of the complex roles of liver macrophages in both protective and harmful responses, these cells form an interesting but difficult cellular target for treatment options in liver diseases. Future efforts should therefore focus on identifying the characteristics of the specific macrophage subpopulations that exert the distinctive functions of interest and on identifying the underlying mechanisms of actions.

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Chapter 11
Intestinal Macrophages: Specialized Antigen-Presenting Cells at the Front Line

Timothy L. Denning and Bali Pulendran

11.1 Introduction

Mammals are eukaryotic organisms, yet they can harbor up to hundreds of trillions of prokaryotes. The majority of these prokaryotes are bacteria that reside within the alimentary/intestinal tract. During the course of evolution these bacteria (collectively referred to as the microbiota) have established a mutualistic relationship with the mammalian host. For the host to reap maximal benefits provided by the microbiota, the mucosal immune system must react in a tolerogenic manner towards these beneficial bacteria, while remaining poised to respond to pathogens. Macrophages are specialized antigen-presenting immune cells located directly beneath the epithelial layer in the intestine, which play a key role in directing innate and adaptive immune responses towards tolerance or inflammation depending on the context. Below we summarize several important features of intestinal macrophages with special emphasis on their phenotypes and functions during health and disease.

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11.2 Phenotype of Steady-State Intestinal Macrophages

In order to understand the biology of intestinal macrophages it is important to focus on unique parameters that distinguish them from other cells in the local microenvironment. Unfortunately, this has been and continues to be an area of considerable debate within the mucosal immunology field (Bain and Mowat 2011; Mowat and Bain 2011; Platt and Mowat 2008). Originally, mouse intestinal macrophages were defined using unique structural features including large phagocytic vacuoles in the cytoplasm (Deane 1964). They were subsequently shown to strongly adhere to plastic and to label with an antibody recognizing the "macrophage-specific" F4/80 antigen (Hume et al. 1983; Pavli et al. 1990). With the advent of multi-color flow cytometry, intense characterization of macrophages, dendritic cells (DCs), eosinophils, and other cell types using different cell surface markers has led to confusion and debate about how to accurately and specifically define these subsets in the intestine, in particular (Hume 2008). The expression of the alpha x integrin, CD11c, on intestinal antigen-presenting cells has been especially problematic when used to identify macrophages and DCs. CD11c is well appreciated to specifically mark DCs but not macrophages in peripheral lymphoid organs (Pulendran et al. 2001), and this has led to the assumption that CD11c can also be used to distinguish DCs from macrophages in the intestine. However, intestinal macrophages constitutively express moderate to high levels of CD11c (Denning et al. 2007, 2011; Pulendran et al. 2008), thus precluding the use of CD11c as a sole marker to distinguish macrophages and DCs in the intestine. CD11b is another marker that has been problematic in distinguishing intestinal macrophages and DCs. While intestinal macrophages almost universally express CD11b, a major subset of intestinal DCs also expresses CD11b, as do neutrophils and eosinophils. The chemokine receptor CX3CR1 initially gained a tremendous amount of attention when it was reported to be specifically expressed by intestinal DCs and to be involved the extension of trans-epithelial dendrites, sampling of luminal antigens, and the clearance of entero-invasive pathogens (Niess et al. 2005). In this study, CD11b and CD11c were used to define the intestinal DCs that expressed CX3CR1, thus, given the aforementioned limitations of these markers, whether these cells were truly DCs or macrophages was not clear. Shortly after CX3CR1 was reported to be expressed by intestinal DCs, interest in CD103 as a marker for intestinal DCs was re-ignited (Brenan and Puklavec 1992; Kilshaw 1993; Johansson-Lindbom et al. 2005). This led to the definition of two functionally non-overlapping subsets of intestinal antigen cells that both express CD11c and major histocompatibility complex (MHC) II: CD103+CX3CR1− and CD103−CX3CR1+ cells (Denning et al. 2007; Schulz et al. 2009). Pabst and colleagues demonstrated that intestinal CD103+CX3CR1− cells could be further divided into CD11b+ and CD11b− subsets with both displaying classic features of DCs such as CCR7-dependent homing to mesenteric lymph nodes, potent antigen-presenting ability to naïve T cells, and imprinting of gut homing markers on naïve T cells. Alternatively, CD103−CX3CR1+ cells did not display steady-state migration to mesenteric lymph nodes, were relatively weak stimulators of naïve T cells,

Table 11.1 Antigens expressed by major subsets of intestinal macrophage and DC cells, and eosinophils, in C57BL/6J mice

Major subsets of intestinal APCs	Antigen expression								
	CD45	MHCII	CD11b	CD11c	F4/80	CD103	CX3CR1	CD64	Siglec F
CD11b– DCs	+++	++	–	+++	–	++	–	–	–
CD11b+ DCs	+++	++	++	+++	–	++	–	–	–
CD11c– Mϕ	+++	+++	++	–/+	++	–	+++	+++	–
CD11c+ Mϕ	+++	+++	++	++/+++	++	–	+++	+++	–
Eosinophils	+++	–	++	++	+++	–	+	–	++

and failed to induce gut homing markers on naïve T cells (Schulz et al. 2009). A thorough characterization of many of these markers revealed that steady-state intestinal lamina propria macrophages are most accurately identified as CD45+MHCII+CD11b+CD11c(+/–) F4/80+CD103–CX3CR1+ cells, while intestinal lamina propria DCs are most accurately identified as CD45+MHCII+ CD11b(+/–)CD11c+F4/80–CD103+CX3CR1– cells (Denning et al. 2011) (Table 11.1). Intestinal macrophages may be divided into those expressing high levels of CD11c, which likely represent lamina propria macrophages, and those expressing low to intermediated levels of CD11c, which likely represent serosal macrophages (Bogunovic et al. 2009). Similarly, intestinal lamina propria DCs may be further divided by expression of CD11b. Intestinal CD11b+ DC do not express CD8α, while CD11b– DC express high levels of the homodimeric form of CD8α, CD8αα, especially in the small intestine (Bilsborough et al. 2003). Of note, the definition of these intestinal subsets as "macrophages" versus "DCs" has been recently validated by the Immunological Genome Consortium, which conducted a detailed gene expression analysis of numerous macrophage and DC populations isolated from various tissues (Gautier et al. 2012). CD64 and Siglec F are two additional markers that deserve inclusion in the above-mentioned panels. The high affinity IgG receptor FcγR, CD64, is highly expressed by CX3CR1+ intestinal macrophages and can be used to further distinguish intestinal macrophages from DCs (Gautier et al. 2012; Tamoutounour et al. 2012; De Calisto et al. 2012). Unlike macrophages and DCs, eosinophils are not typically classified as antigen-presenting cells, however, they are abundant in the intestine and have been confused with macrophages due to the overlapping expression of several antigens (Mowat and Bain 2011). Both eosinophils and macrophages express CD11b and F4/80, which has necessitated analysis of three additional parameters to distinguish them from one another: side scatter (SSC) properties, expression of MHCII, and expression of Siglec F. Intestinal eosinophils display high SSC and express Siglec F, but not MHCII, while intestinal macrophages display low SSC and high levels of MHCII, but not Siglec F (Carlens et al. 2009). Additional antigens such as CD68 (macrosialin) are not commonly analyzed, but may be useful in defining intestinal macrophages when combined with additional markers defined above.

Unfortunately, the markers used to define intestinal macrophages in mice are not directly translatable to humans. In humans, several markers including HLA-DR, CD68, and CD13 are used to define steady-state intestinal macrophages. Interestingly, human intestinal macrophages do not express CD11a, CD11b, CD11c, CD14, CD18, CD89, or FcγRI–III (CD64, CD32, CD16), whereas these receptors are strongly expressed on human blood monocytes (Smith et al. 2001, 2005, 2011; Smythies et al. 2005). Therefore, intestinal macrophages in mice and humans display unique cell surface antigens that can be used to distinguish them from other intestinal antigen-presenting cells. In summary, the most accurate way to distinguish intestinal macrophages, DCs, and eosinophils whether they are of mouse or human origin is by performing multi-color flow cytometry using the appropriate combination of antibodies that recognize uniquely expressed cell surface antigens.

11.3 Origin and Development of Intestinal Macrophages

Monocytes, lymphoid tissue DCs, and plasmacytoid DCs (pDCs) all share a common bone marrow progenitor termed the macrophage and DC precursor (MDP) (Fogg et al. 2006). MDPs can differentiate into the common DC precursor (CDP) that is restricted to producing lymphoid organ DCs and pDCs, but not monocytes. The CDP can then produce pre-DCs, which are the progenitors that have lost the ability to differentiate into pDCs and instead give rise to peripheral DCs, including intestinal CD103+ DCs, in a FMS-like tyrosine kinase 3 (Flt3) dependent manner (Fig. 11.1). Monocytes follow a different developmental program from DCs. Most tissue macrophages are derived from circulating blood monocytes, however there exists two major subsets of murine blood monocytes that differentially give rise to macrophages in non-inflamed versus inflamed tissues (Geissmann et al. 2003). The original description of these two blood monocyte subsets suggested that the CX3CR1(lo)CCR2+Gr1+ subset contains short-lived cells that are actively recruited to inflamed tissues, while the CX3CR1(hi)CCR2−Gr1− subset is characterized by CX3CR1-mediated recruitment to non-inflamed tissues, including the intestine. Thus, the CX3CR1(hi)CCR2−Gr1− subset of blood monocytes was considered the precursor population of intestinal macrophages residing in the normal intestine. However, adoptive transfer studies later demonstrated that granulocyte-macrophage colony stimulating factor (GM-CSF) dependent Ly6C(hi), but not Ly6C(lo), monocytes can give rise to both inflammatory CX3CR1(lo) macrophages/DCs as well as anti-inflammatory, resident intestinal CX3CR1(hi) macrophages (Bogunovic et al. 2009; Varol et al. 2009). These seemingly discrepant observations may be explained by a recent fate mapping study that revealed $Ly6C^+$ blood monocytes are obligatory precursor cells of $Ly6C^-$ blood monocytes (Yona et al. 2013). Interestingly, $Ly6C^+$ blood monocytes were shown to negatively regulate the $Ly6C^-$ blood monocyte compartment via acting as a CSF-1 "sink." These data suggest that a critical balance between $Ly6C^+$ and $Ly6C^-$ blood monocytes influences the overall intestinal macrophages pool (Yona et al. 2013). In the steady state, CX3CR1+$Ly6C^-$ blood

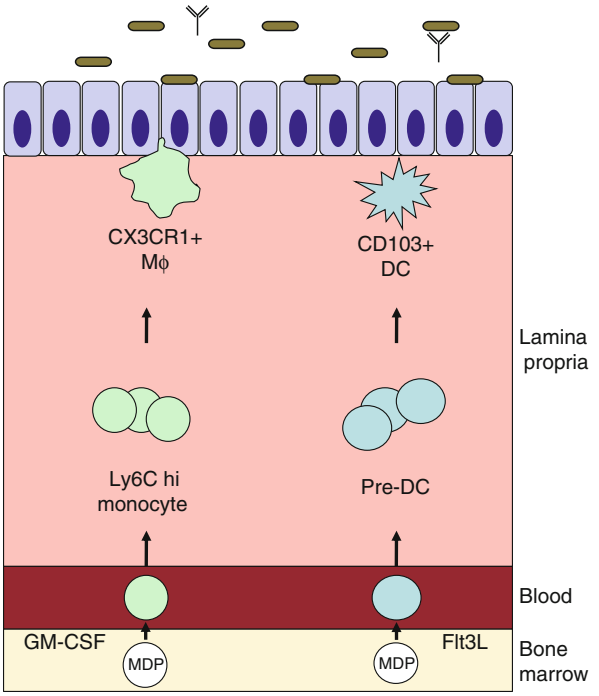

Fig. 11.1 Macrophage and DC precursor (MDP) can differentiate into the common DC precursor (CDP). The CDP can then produce pre-DCs, which are the progenitors that give rise to intestinal CD103+ DCs, in a FMS-like tyrosine kinase 3 (Flt3) dependent manner. Alternatively, granulocyte-macrophage colony stimulating factor (GM-CSF) dependent Ly6C(hi) monocytes give rise to CX3CR1+ anti-inflammatory, resident macrophages

monocytes may be the major source of intestinal macrophages, while during inflammation Ly6C$^+$ blood monocytes may become the dominant precursor due to their expansion combined with negative regulation of Ly6C$^-$ blood monocytes. It is also possible that Ly6C$^+$ blood monocytes are the major precursors for steady-state intestinal macrophages the intestine, since it is always in a state of controlled "inflammation." Consistent with this concept is the idea that intestinal epithelial cells constitutively produce CX3CL1, the ligand for CX3CR1, perhaps in response to sensing of the intestinal microbiota (Niess et al. 2005; Muehlhoefer et al. 2000). This could allow for the recruitment of CX3CR1+ blood monocytes into the intestine where they differentiate into resident intestinal macrophages that continue to express high levels of CX3CR1. Indeed, we have recently shown that loss of CX3CR1 or CX3CL1 in mice resulted in a dramatic reduction in the number of intestinal macrophages in the small and large intestines (Medina-Contreras et al. 2011). These data suggest that the CX3CR1/CX3CL1 axis regulates the pool of intestinal macrophage in the steady state. CCR2 is another chemokine receptor expressed by a subset of resident intestinal macrophages, which may be involved in their recruitment/retention. A specific subset of IL-10 producing intestinal macrophages appears uniquely dependent upon monocyte chemotactic protein-1 (CCL2), one of the ligands for CCR2 (Takada et al. 2010). Other factors such as TGF-β and IL-8 are produced by intestinal stromal cells and can induce monocyte chemotaxis (Smythies et al. 2005; Smith et al. 2011), however the role of these cytokines in the recruitment/retention of intestinal macrophages is not clear.

Beyond replenishment from blood monocytes, intestinal macrophages may also be regulated by self-renewal. Macrophages can be found within the intestine even prior to birth and these earliest precursors may be derived from the yolk sac. The specific mechanisms that regulate the migration and/or expansion of this first wave of intestinal macrophages are not entirely clear, but likely involve CSF-1. Deficiency in CSF-1 or antibody-mediated blockade of CSF-1R resulted in the specific absence of intestinal macrophages, suggesting that CSF-1 may be required for the local maintenance and/or expansion of the earliest precursors of intestinal macrophages (Bogunovic et al. 2009; MacDonald et al. 2010; Ginhoux et al. 2010). Such a process of self-renewal has been well defined in other macrophage populations including microglia in the brain and Kupffer cells in the liver (Ginhoux et al. 2010; Geissmann et al. 2010; Ajami et al. 2007). Overall, the relative contribution of self-renewal versus recruitment of blood monocytes to the overall pool of resident intestinal macrophages in the adult is an important issue that requires further investigation.

11.4 Steady-State Functions of Intestinal Macrophages

As one might predict from their positioning directly beneath the epithelial layer, intestinal lamina propria macrophages are poised to react to components of the microbiota that they come into contact with. Studies in both mice and humans have demonstrated that intestinal macrophages are avidly phagocytic, which is consistent with the large phagocytic vacuoles present in their cytoplasm, and have strong bactericidal activity (Smythies et al. 2005). These "housekeeping" functions of intestinal macrophages may play a critical role in maintaining homeostasis in the gut, since mice with a reduction in intestinal macrophages due to CX3CR1 or CX3CL1 deficiency display increased translocation of bacteria to mesenteric lymph nodes and are more susceptible to experimental colitis (Medina-Contreras et al. 2011). This concept is further supported by data demonstrating that CX3CR1+ DCs/macrophages can extend trans-epithelial processes and sample luminal antigens and influence the clearance of entero-invasive pathogens (Niess et al. 2005). CX3CR1 expressing intestinal DCs/macrophages are also involved in the surveillance of circulatory antigens and can act as a sort of conduit for the processing of intestinally absorbed antigens and self-antigens (Chang et al. 2013). In addition to phagocytosing bacteria and absorbed antigens, intestinal macrophages may also engulf apoptotic cells since they express the scavenger receptor CD36 (Mowat and Bain 2011).

Upon interaction with bacterial ligands, intestinal macrophages react very differently than macrophages found in other peripheral sites. In vitro studies have demonstrated that intestinal macrophages do not produce inflammatory cytokines such as IL-1, -6, -12, -23, or TNF in response to a wide array of toll-like receptor (TLR) ligands (Bain and Mowat 2011; Denning et al. 2007; Smith et al. 2001, 2011; Smythies et al. 2005; Kamada et al. 2005). In mice, this "inflammatory anergy" appears to be explained by their low expression of TLRs and associated signaling machinery, as well as constitutive production of high levels of IL-10, which are

unique features of intestinal macrophages that are not shared with intestinal DCs. Stimulation of intestinal macrophages with TLR ligands while blocking IL-10 signaling was able to reverse this hyporesponsiveness and similar results were obtained using IL-10 deficient macrophages (Denning et al. 2007). Interestingly, human intestinal macrophages also display profound inflammatory anergy, yet do not spontaneously secrete IL-10 (Smythies et al. 2005; Smith et al. 2011). This suggests that other factors regulate the hyporesponsiveness of human intestinal macrophages towards bacteria. Among the likely candidates, TGF-β production by intestinal stromal cells has been implicated in this hyporesponsiveness (Smythies et al. 2005; Smith et al. 2011), as has PPAR-γ (Jiang et al. 1998; Shah et al. 2007). Thus, intestinal macrophage function may be controlled via conditioning by microbiota and local stromal cells.

In addition to their innate hyporesponsiveness, intestinal macrophages can also promote tolerogenic adaptive immune responses. Originally described to act as weak stimulators in allogeneic mixed lymphocyte reactions (MLRs) despite high expression of class II major histocompatibility complex antigens, intestinal macrophages were further shown to induce indomethacin-sensitive suppression in MLRs (Pavli et al. 1990). More recently, intestinal macrophages have been implicated in the induction (Denning et al. 2007), maintenance (Murai et al. 2009), and expansion (Hadis et al. 2011) of CD4+Foxp3+ regulatory T cells and the control of CD8+ T cell activation (Chang et al. 2013). This ability to induce regulatory T cells is largely mediated by IL-10, TGF-β, and the vitamin A metabolite retinoic acid (RA). Like intestinal DCs, intestinal macrophages express retinaldehyde dehydrogenase (RALDH) enzymes that are critical for the generation of RA (Manicassamy and Pulendran 2009). During co-culture with CD4+ T cells, intestinal macrophages promote induced regulatory T cells generation in an IL-10 and RA-specific manner (Denning et al. 2007). IL-10 and RA also appear to be involved in promoting Foxp3+ regulatory T cell abundance in vivo (Murai et al. 2009; Hadis et al. 2011; Hill et al. 2008). In humans, intestinal macrophages express CCL20 (MIP3α), the ligand for CCR6, which is expressed on CD4+ regulatory T cells and may lead to close interactions between these cells in the healthy intestine. Additionally, intestinal macrophages can promote tolerance by suppressing IL-17 producing CD4+ T cell responses in vitro and in vivo (Denning et al. 2007; Medina-Contreras et al. 2011). This may be the result of directly or indirectly inhibiting the Th17-promoting functions of intestinal CD11b+ DCs (Denning et al. 2007). The location where intestinal macrophages promote Foxp3+ regulatory T cells and inhibit Th17 cells is most likely the lamina propria since these cells do not express CCR7 or migrate to the mesenteric lymph nodes, as intestinal CD103+ DCs do (Hadis et al. 2011). Thus, intestinal macrophages regulate themselves and neighboring immune cells directly in the lamina propria where these regulatory mechanisms are necessary to prevent pathological inflammation (Fig. 11.2).

Given the critical role of resident intestinal macrophages in regulating tolerance towards bacterial ligands and dampening adaptive immune reactivity, one would predict that loss or dysfunction of these cells would have negative consequences on homeostasis in the gut. In fact, several animal models highlight a central role for

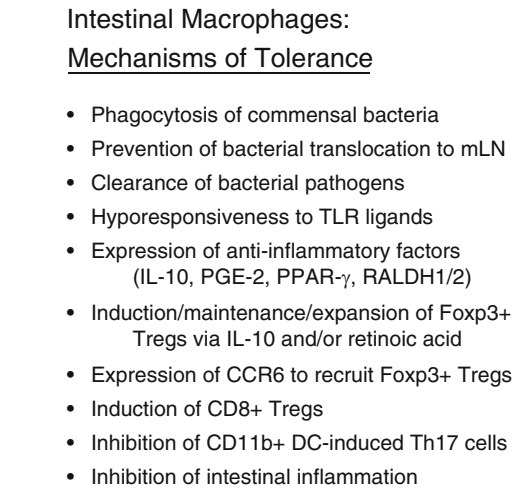

Fig. 11.2 Tolerogenic mechanisms employed by steady-state, anti-inflammatory intestinal macrophages

resident intestinal macrophage function in oral tolerance and prevention of intestinal inflammation. Deficiency in F4/80, which is highly expressed by intestinal macrophages, results in the loss of tolerance to orally administered antigens and defective generation of efferent CD8+ regulatory T cells that suppress antigen-specific DTH responses (Lin et al. 2005). It is not clear that the abrogation of oral tolerance in F4/80 deficient mice is related to loss of intestinal macrophages, however, since F4/80 is not required for the development or tissue distribution of tissue macrophages. Similar to F4/80, deficiency of CD11b results in defective oral tolerance that is associated with enhanced Th17 responses (Ehirchiou et al. 2007). Expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) by resident intestinal macrophages is another means via which these cells can regulate intestinal homeostasis. PPAR-γ can inhibit pro-inflammatory cytokine secretion and restrict CCR-2 mediated migration of pro-inflammatory monocytes into the intestine. Further, macrophage-specific deletion of PPAR-γ in mice leads to intestinal macrophage activation and exacerbation of intestinal inflammation (Shah et al. 2007). Likewise, delivery of PPAR ligands has been shown to ameliorate experimental colitis (Su et al. 1999; Dubuquoy et al. 2006). Cyclo-oxygenase 2-dependent production of prostaglandin E2 by intestinal macrophages may also prevent intestinal inflammation by direct effects on the epithelia stem cell niche (Pull et al. 2005). Additionally, CD200 receptor 1 (CD200R1) has been suggested as a negative regulatory surface marker on macrophages, but preliminary data was unable to reveal any role for this molecule in experimental colitis or intestinal macrophage function (Bain and Mowat 2011).

Some of the earliest direct evidence for an involvement for resident macrophages in regulating experimental colitis was obtained using the clodronate liposome mediated depletion strategy (Qualls et al. 2006). When mice were pretreated with clodronate liposomes to deplete intestinal macrophages, a surprising enhancement of colitis was observed. Similar results were also seen using a conditional system that induces apoptosis of macrophages in vivo (MaFIA mice) (Qualls et al. 2006).

Depletion of intestinal macrophages using clodronate liposomes can also inhibit the protective effects of helminthes on experimental colitis, which may be via loss of macrophage-derived IL-10 production (Hunter et al. 2010). Resident intestinal CX3CR1+ macrophages also play a role in ameliorating chronic colitis. The co-transfer of CX3CR1+ intestinal macrophages into Rag-deficient mice receiving CD4+CD45RB(hi) T cells was able to significantly inhibit intestinal inflammation. Interestingly, the transferred macrophages could home back to the intestine where they may have mediated their protective effects (Kayama et al. 2012). We have also demonstrated a protective effect of CX3CR1+ intestinal macrophages in the dextran-sodium sulfate (DSS) model of acute colitis. We observed that deficiency in CX3CR1 or CX3CL1 resulted in dramatically enhanced Th17 driven colitis, which was reversed by the transfer of CX3CR1+ macrophages (Medina-Contreras et al. 2011). Collectively, these data support the concept that resident intestinal macrophages have important anti-inflammatory properties, which are beneficial in maintaining homeostasis at the mucosal border.

Intestinal macrophages may also play a role in mucosal wound healing and in the resolution of intestinal inflammation. Using an in vivo acute injury model created by taking a biopsy of the colonic mucosa, Trem2 expressing macrophages were shown to contribute to epithelial proliferation, suppression of pro-inflammatory cytokine production, and closure of the wound bed (Pull et al. 2005). Trem2 is a cell surface marker specifically induced in alternatively activated (M2) macrophages by IL-4 and IL-13, which functions to activate Stat6 and arginase expression. Intestinal macrophage-derived arginase may thus shift L-arginine utilization towards polyamine production resulting in epithelial proliferation. M2 macrophages have also been reported to significantly reduce the severity of experimental colitis by increasing collagen deposition and secreting IL-10 (Hunter et al. 2010). Therefore, yet another beneficial function of intestinal macrophages it to regulate wound healing repair.

11.5 Intestinal Macrophages and the Microbiota

As mentioned at the outset, intestinal bacteria dramatically influence the host, in particular the mucosal immune system. The number of intestinal bacteria is believed to be approximately 10^{12} organisms/ml of luminal contents in the colon alone. The host immune system is not ignorant of these bacteria as there is constitutive steady-state sampling of luminal bacteria and some bacteria are adept at penetrating the thick mucus layer and coming into direct contact with intestinal epithelial cells and underlying immune cells, in particular intestinal macrophages. The role of the microbiota in the recruitment of macrophages to the intestinal mucosa is not entirely clear. Macrophages clearly populate the intestine before birth (Lotz et al. 2006) when colonization of the intestine with bacteria has yet to take place, suggesting that the initial wave of intestinal macrophages is independent of the microbiota. These immature macrophages are already hyporesponsive to TLR agonists, perhaps as a result of TGF-β produced by intestinal stromal cells (Maheshwari et al. 2011).

Colonization of the intestine with bacteria does however influence the subsequent expansion of this initial pool of macrophages or migration of new precursors into the gut, as evidenced by a strong correlation between bacterial abundance and macrophage number along the length of the gastrointestinal tract (Lee et al. 1985). Additionally, macrophages are reduced in number in the intestines of germ-free mice (Niess and Adler 2010).

Beyond macrophage expansion and/or recruitment to the intestine, sensing of the microbiota is critical for several unique features of these cells (Bain and Mowat 2011; Mowat and Bain 2011). Intestinal macrophages that cannot sense bacteria via MyD88-dependent signaling have severely reduced expression of IL-10 and IL-10-inducible genes including the hemoglobin scavenger receptor, CD163 (Ueda et al. 2010). Thus, the anti-inflammatory program adopted by intestinal macrophages is in response to sensing of the microbiota and may control the inflammatory anergy that is a hallmark of these cells. This idea has been supported by the demonstration that macrophages isolated from the intestines of germ-free mice display dramatically enhanced pro-inflammatory cytokine responses to LPS when compared to specific pathogen free mice (Ueda et al. 2010).

In light of their avidly phagocytic nature, after intestinal macrophages encounter bacteria they likely present antigens to T cells via MHCII molecules. However, they do not express CCR7 and are thus incapable of migrating to the mesenteric lymph nodes to interact with naïve T cells in this site, as intestinal DCs do (Schulz et al. 2009). Therefore, the T cell stimulatory function of intestinal macrophages must take place directly in situ. Interestingly, a recent study showed that the microbiota is responsible for restricting the migration of CX3CR1+ macrophages from the intestine to the mesenteric lymph node (Diehl et al. 2013). In antibiotic treated mice, or mice deficient in MyD88, CX3CR1+ intestinal macrophages were shown to phagocytose bacteria, upregulate CCR7, and migrate to the mesenteric lymph nodes where they induce T cell responses and promote differentiation of IgA producing B cells. Additional data defining the physiological circumstances when CX3CR1+ intestinal macrophages may migrate to the mesenteric lymph node should provide further insight into the dichotomous role of intestinal macrophages and DCs.

11.6 Intestinal Macrophages and Inflammation

During human and experimental inflammatory bowel disease (IBD), the intestinal epithelial barrier becomes disrupted leading to a massive influx of bacteria into the underlying lamina propria and overt immune cell infiltration and activation (Abraham and Medzhitov 2011). Blood monocytes are one of the major leukocyte subsets recruited to the inflamed intestine where they differentiate into "inflammatory" macrophages. Interestingly, these inflammatory macrophages are derived from the same Ly6C$^+$ blood monocyte precursors that resident anti-inflammatory macrophages are derived from, thus highlighting that the inflammatory status of the intestine ultimately determines whether infiltrating Ly6C$^+$ monocytes will become anti- or pro-inflammatory macrophages (Rivollier et al. 2012).

Inflammatory macrophages express high levels of Ly6C, CCR2, TLR2 and triggering receptor expressed by myeloid cells-1 (TREM1), and intermediate to low levels of CX3CR1 and MHCII making them easily distinguishable from CX3CR1+CCR2−TLR2−TREM1−MHCII(hi) resident macrophages (Bain and Mowat 2011). After encounter with the flood of bacterial ligands and activation of MyD88-dependent signaling cascades (Pulendran et al. 2001), these cells produce high levels of the pro-inflammatory cytokines IL-1, -6, -8, -12, -23, TNF, and express iNOS (Rivollier et al. 2012; Zigmond et al. 2012; Platt et al. 2010). These inflammatory mediators can contribute to tissue pathology via upregulation of adhesion molecules on the vascular endothelium, increasing epithelial permeability, enhancing recruitment of mononuclear and granulocytic cells, activating DCs, inducing the differentiation of Th1 and Th17 cells, and promoting tissue degradation and fibrosis (Fig. 11.3).

With such a potent inflammatory profile, specific targeting of inflammatory macrophages has been an attractive idea for the possible prevention or treatment of intestinal inflammation. In mice, administration of neutralizing anti-CCR2 antibody results in near complete prevention of inflammatory macrophage differentiation in the intestine, reduction in pro-inflammatory cytokines, and less severe acute colitis

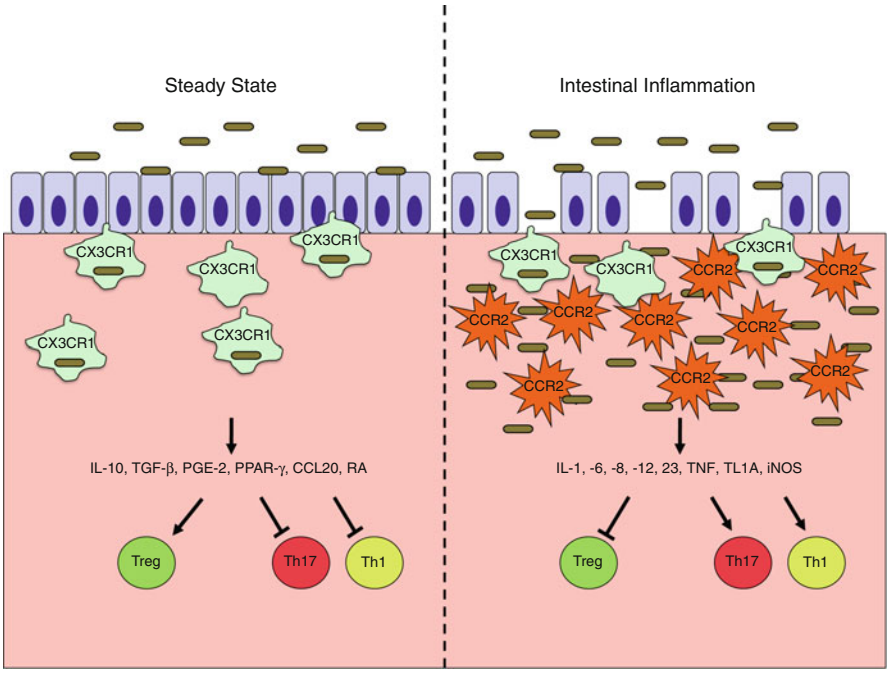

Fig. 11.3 During steady-state conditions, CX3CR1+ macrophages predominate in the intestinal lamina propria. These cells are avidly phagocytic and produce anti-inflammatory/pro-regulatory factors that promote the differentiation, maintenance, and expansion of Foxp3+ regulatory T cells while inhibiting the differentiation of Th17/Th1 cells. Under inflammatory conditions the intestinal epithelial barrier is disrupted, bacteria translocate more freely into the lamina propria, and Ly6C$^+$ CCR2+ macrophages predominate. Ly6C$^+$ CCR2+ macrophages secret pro-inflammatory factors and drive the differentiation of Th17/Th1 cells while inhibiting Foxp3+ T cell responses

(Zigmond et al. 2012). These data are further supported by observations that CCR2 deficient mice are resistant to acute and chronic models of colitis (Zigmond et al. 2012; Platt et al. 2010). In human IBD, macrophages are also believed to contribute to disease pathology. A unique population of CD14+ intestinal macrophages was recently identified as a possible cell type contributing to the pathogenesis of Crohn's disease (Kamada et al. 2008). These macrophages are dramatically increased in diseased tissue and produce high levels of IL-23 and TNF, and induce IFN-γ secretion by lamina propria mononuclear cells. This may create a sort of feed-forward loop whereby IL-23 induced IFN-γ secretion further activates inflammatory macrophages to secrete even more IL-23 and augment inflammation. Additionally, in Crohn's disease patients, CD14+CD209+ intestinal macrophages express MHCII and exhibit potent antigen-presenting capacity and induce Th17 cells (Kamada et al. 2009). The exact mechanism(s) by which intestinal macrophages mediate this plethora of pro-inflammatory effects are not clear, but may involve macrophage expression of TREM-1 and their secretion of the tumor necrosis factor like protein TL1A (Takedatsu et al. 2008; Shih et al. 2009; Kamada et al. 2010).

Genome wide association studies in Crohn's disease and ulcerative colitis patients have identified numerous single nucleotide polymorphisms in genes associated with intestinal macrophage function. The identification of these polymorphisms may provide further insight into how to manipulate resident and inflammatory macrophage function to improve the health of IBD patients (Abraham and Medzhitov 2011; Stappenbeck et al. 2011; Rosenstiel et al. 2009). In particular, the intracellular pattern recognition receptor NOD-2 is expressed in intestinal macrophages and recognized the bacterial cell wall component muramyl dipeptide. Approximately 30 % of Crohn's disease patients with small intestinal inflammation have a non-functional mutation in the NOD-2 gene, which is the strongest genetic linkage discovered thus far for human IBD (Hugot et al. 2001; Ogura et al. 2001; Cho and Weaver 2007). This mutation in NOD-2 may lead to hyperresponsive macrophages that contribute to intestinal inflammation. Recent experimental evidence demonstrated that loss of NOD-2 ameliorates spontaneous colitis in IL-10 deficient mice (Jamontt et al. 2013). Thus, there may be a crosstalk between NOD-2 and IL-10 that dictates intestinal macrophage function. Several autophagy related genes including the leucine rich repeat kinase 2 (LRRK2), ATG16L1, and the immunity related GTPase family M (IRGM) have also been identified by GWAS to confer susceptibility to Crohn's disease (Stappenbeck et al. 2011; Cho and Weaver 2007). LRRK2, Atg16L1, and IRGM are expressed by intestinal macrophages and dysfunction in these proteins may affect normal autophagy related functions that are required to maintain intestinal homeostasis.

11.7 Conclusion

In summary, intestinal macrophages are a complex population of cells that serve critical functions in both health and disease. From birth, resident intestinal macrophages patrol the front lines of the intestine, quickly forging a tolerogenic

relationship with the microbiota. They potently regulate their own reactivity while concomitantly expressing factors that suppress other immune cells. If they become overwhelmed during infection or inflammation, their precursors begin to differentiate into a subset of inflammatory macrophages that do not tolerate the microbiota. Further understanding what controls resident and inflammatory macrophage functions in the steady state and during inflammation will afford the opportunity to manipulate these cells for therapeutic purposes.

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Part IV
Macrophages in Pathology

Chapter 12
The Wound Macrophage

Meredith J. Crane and Jorge E. Albina

12.1 Introduction

The ability to heal wounds through exact tissue regeneration and without the formation of a collagenous scar is limited in mammalian adults to the repair of superficial epithelial injuries not involving the underlying stroma, and to wounds in early fetal development. In all other circumstances, the resolution of injury through "replacement-by-scar" has been preserved in evolution and applies to virtually all soft tissues in mammals. A healed wound is the end product of the activities of cells that constitute what can be conceptually described as a "wound organ." This transient "neo-organ" is assembled at the time and site of injury and disassembled once repair is complete, leaving in place a scar as evidence of the repair process. The wound organ parenchyma is comprised of a temporally changing assembly of inflammatory cells, neovessels, fibroblasts, myofibroblasts, regenerating nerves, and other cells specific to the site of the injury (i.e., keratinocytes, osteocytes, hepatocytes, etc.).

12.2 Macrophages and the Regulation of Wound Healing

The concept that macrophages are unique among blood-borne components of the wound organ in being essential to normal repair was originally predicated from findings in guinea pigs where macrophage depletion using anti-macrophage antiserum and glucocorticoids reduced the rate of cutaneous wound healing

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(Leibovich and Ross 1975). Strong confirmatory evidence for the essential role of macrophages in normal repair has more recently been provided by studies in genetically modified mice depleted of monocytes and macrophages. The elimination of macrophages via the administration of diphtheria toxin to transgenic mice expressing the diphtheria toxin receptor under the control of the lysozyme M (Lucas et al. 2010; Goren et al. 2009) or the CD11b promoter (Mirza et al. 2009) resulted in abnormal wound healing. The alterations in the repair process in these animals included reductions in wound re-epithelialization, contraction, neovascularization, and granulation tissue formation.

Lucas et al., using the aforementioned transgenic mice, further defined the contribution of macrophages to the healing process by administering diphtheria toxin at different time points in relation to excisional skin wounding. Using this strategy, macrophages were depleted either during the initial inflammatory response to injury (first 4 days after injury), the intermediate phase of granulation tissue formation (4–10 days after injury), or during the consolidation of fibrosis (10–14 days post injury). Macrophage depletion during the initial phase of repair resulted in severely altered wound healing with reductions in all components of the healing response, including granulation tissue formation, epithelialization, and scar deposition. Macrophage ablation during the intermediate period was associated with wound hemorrhage, increased apoptosis of neo-vascular endothelial cells, and collapse of the developing vascular structure that characterizes granulation tissue formation. The authors ascribed these alterations to reductions in TGF-β and VEGF in the macrophage-depleted wounds. Macrophage ablation, once the fibrotic phase of repair was ongoing, was without effect (Lucas et al. 2010).

12.3 Wound Macrophages and the Regulation of Fibrosis

Inflammation is a prerequisite for healing with a scar. Evidence linking TGF-β production by wound macrophages and scar formation is convincing. Cutaneous wounds in embryos prior to the development of the myeloid system do not elicit an inflammatory response, lack TGF-β, and heal without scar (Buchanan et al. 2009). Moreover, TGF-β signaling deficient SMAD3 knockouts recruit macrophages poorly into skin wounds and also heal without scar (Ashcroft and Roberts 2000). These observations are not restricted to cutaneous wounds. Lung injury in SMAD3 knockouts results in decreased procollagen mRNA expression and fibrosis, and the overexpression of TGF-β1 increases fibrosis in wild type, but not in SMAD3-deficient, mice (Bonniaud et al. 2004). Because wound macrophages have been demonstrated to produce and release TGF-β, this growth factor appears to fulfill the criteria of a *bona fide* wound macrophage-derived mediator contributing to wound healing (Daley et al. 2010).

Recent results obtained from wounds in macrophage-depleted mice support the aforementioned conclusion. Mirza et al. (2009) described decreased TGF-β1 mRNA and protein, along with reduced collagen accumulation in wounds in macrophage-deficient mice. Lucas et al. confirmed the reduction in TGF-β1 protein

by histochemistry in macrophage-depleted animals and correlated this reduction with the inability of the mice to deposit granulation tissue and to form a normal scar. The authors proposed, in addition, that TGF-β1 is required for the constitution of a normal vascular network in the healing wound, and supported their conclusion by demonstrating alterations in animals deficient in TGF-β receptor II in myeloid cells that are strikingly similar to those in the macrophage ablated mice (Lucas et al. 2010).

Goren et al. (2009), also using macrophage-depleted mice, found reductions in the number of myofibroblasts and in the rate of wound contraction in macrophage-depleted animals, despite normal TGF-β bioactivity. The discrepancy between findings by these authors and those reported by Lucas et al. (2010) and Mirza et al. (2009) on the expression of TFG-β in very similar models of macrophage depletion and injury may be explained by an uneven distribution of this growth factor in areas critically proximal to sites of myofibroblast differentiation in the wound (Figs. 12.1 and 12.2).

The production of TGF-β by wound macrophages increases as Ly6C^{low} CX3CR1^{high} macrophages accumulate in murine wounds (vide infra), and CX3CR1 expression correlates with the extent of fibrosis in murine kidney and heart (Furuichi et al. 2006; Nahrendorf et al. 2007; Daley et al. 2010). Two single nucleotide polymorphisms coding for non-synonymous amino acid substitutions in *CX3CR1* in

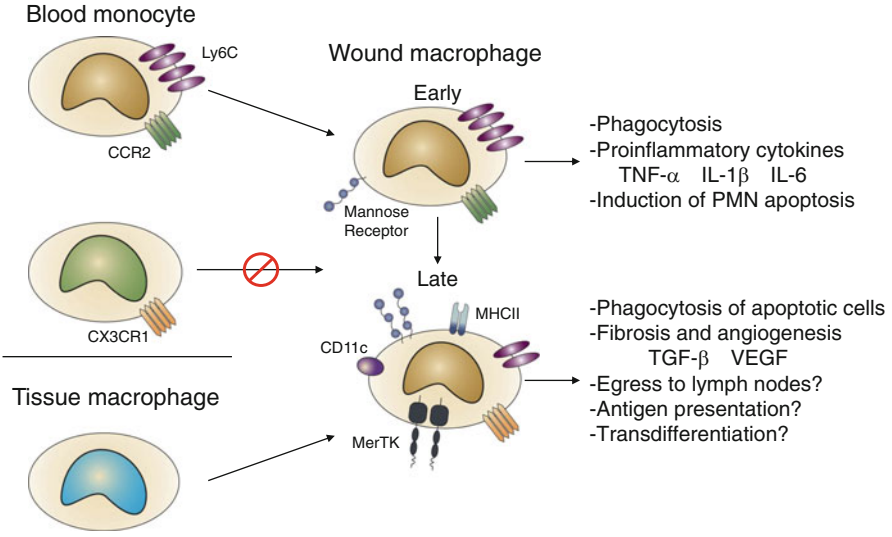

Fig. 12.1 Wound macrophage phenotypes in the mouse. The figure depicts the two sub-populations of murine blood monocytes that are distinguished by their expression of Ly6C and chemokine receptors CCR2 and CX3CR1. Ly6C^{high}CCR2^{high} monocytes are recruited into wounds early after injury and are known to produce pro-inflammatory cytokines and to clear wound debris by phagocytosis. These cells are also likely to induce apoptosis in neutrophils. Ly6C^{low}CX3CR1^{high} macrophages in wounds do not appear to originate from circulating Ly6C^{low}CX3CR1^{high} monocytes but rather from Ly6C^{high}CCR2^{high} monocyte-derived macrophages or from resident tissue macrophages. Ly6C^{low} wound macrophages express mannose receptor (CD206), some dendritic cell markers and MerTK, and produce TGF-β and VEGF rather than pro-inflammatory mediators

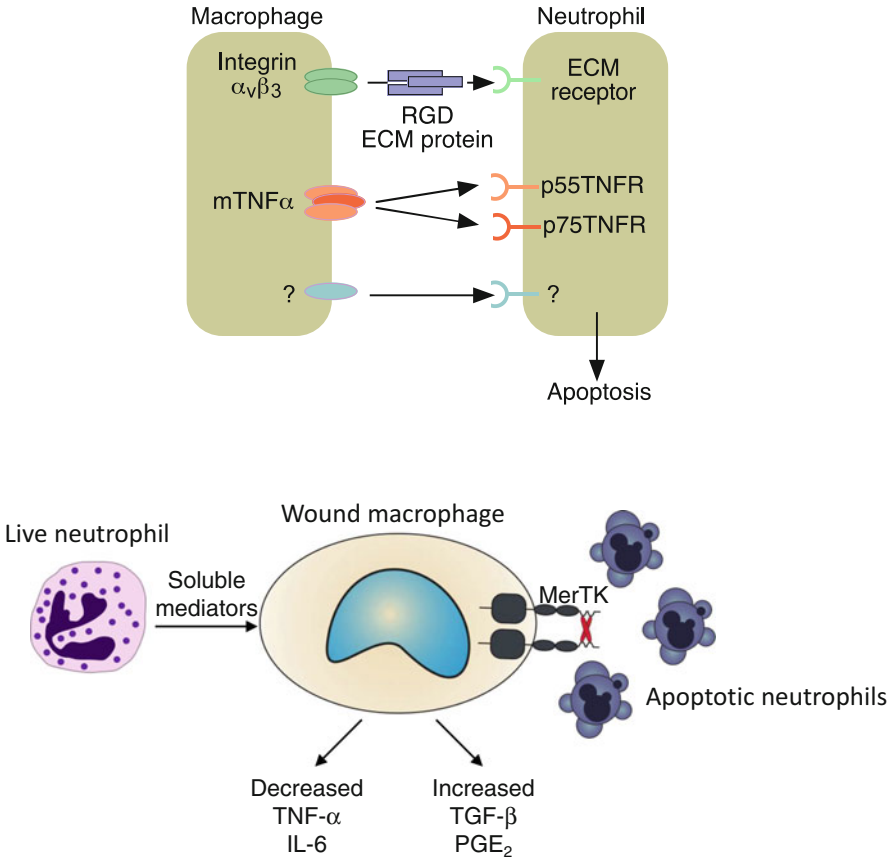

Fig. 12.2 Macrophage/neutrophil interactions in healing wounds. *Top panel*: wound macrophages induce apoptosis in wound neutrophils through a mechanism requiring intercellular binding through a molecular bridge [composed of macrophage integrin $\alpha_v\beta_3$, an RGD-containing extracellular matrix protein, and an extracellular matrix (ECM) receptor on the neutrophil], and the engagement of p55 and p75 TNF-α receptors in the neutrophil by membrane-bound TNF-α (mTNF-α) on the macrophage. The *question marks* in the figure indicate the potential involvement of other apoptosis inducers and their receptors (i.e., FAS/FAS ligand) (Meszaros et al. 2000). *Lower panel*: intact neutrophils release soluble mediators that suppress pro-inflammatory cytokine production by macrophages. In addition, recognition, binding, and phagocytosis of apoptotic neutrophils by macrophages results in an anti-inflammatory phenotype in the macrophage. Apoptotic cell recognition and binding is mediated, at least in part, by macrophage MerTK binding to phosphatidylserine exposed on the surface of the target apoptotic cell using the bridging molecule Gas6 (shown in *red* in the figure)

humans are associated with increased binding of CX3CL1 to its receptor, and correlate with increased hepatic fibrosis in chronic hepatitis C and in a fibro-stenosing form of Crohn's disease (Daoudi et al. 2004; Wasmuth et al. 2008; Brand et al. 2006). Moreover, increased numbers of CX3CR1^{high} monocytes/macrophages have

been found in areas of cutaneous or pulmonary inflammation and fibrosis in patients with systemic sclerosis (Hasegawa et al. 2005). The longer macrophage lifespan reportedly stemming from CX3CR1 signaling may contribute to the prolonged production and release of fibrogenic mediators in sites of chronic inflammation.

Just as macrophages appear to be required for the initiation of scarring, they are also involved in matrix remodeling and in the resolution of fibrosis. Duffield et al. (2005) reported that macrophage ablation during the phase of fibrotic development after the administration of carbon tetrachloride resulted in decreased collagen deposition and myofibroblast accumulation in the liver. In contrast, depleting macrophages during the resolution of fibrosis retarded and reduced the disappearance of scar tissue (Duffield et al. 2005). Because macrophages can induce apoptosis and ingest fibroblasts, myofibroblasts, and vascular endothelial cells, they can regulate the transition between the granulation phase of wound healing and the subsequent deposition of an almost avascular scar (Desmouliere et al. 1995; Diez-Roux and Lang 1997).

12.4 Wound Macrophages and Neovascularization

Evidence obtained in macrophage-deficient mice confirmed the essentiality of macrophages to the neovascularization of wounds. Mirza et al. (2009), Goren et al. (2009), and Lucas et al. (2010) found reduced and abnormal vascularization in their models of macrophage-depleted wounds.

Neovascularization during granulation tissue formation in wounds is controlled by a variety of cytokines and growth factors (Werner and Grose 2003). Members of the VEGF family and their receptors have been shown to be prominently expressed in animal and human wounds, and to be produced there mainly by macrophages and keratinocytes (Brown et al. 1992). Treatment with anti-VEGF A antibodies resulted in marked reductions in neovascularization and granulation tissue formation in porcine wounds (Howdieshell et al. 2001), and an anti-VEGF antibody neutralized the angiogenic activity of human wound fluids in vitro (Nissen et al. 1998).

A connection between angiogenesis and the $Ly6C^{low}CX3CR1^{high}$ wound macrophages that are discussed later on was proposed by Nahrendorf et al. (2007), who showed that these cells produce VEGF, and that their elimination results in reduced neovascularization of ischemic myocardium in mice. Others reported confirmatory results in CX3CR1 knockout mice (Ishida et al. 2008). Wounds in these mice have reduced neovessel density, as well as less VEGF mRNA and protein than those in wild type animals. In contrast, CX3CR1-deficient animals subjected to corneal injuries exhibited increased vascularization, along with decreased local production of anti-angiogenic mediators thrombospondin 1 and 2, and their activator ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motif-1) (Lu et al. 2009). It is possible that the role of wound macrophages in angiogenesis varies with injury site. It is more likely that the cells provide pro- and anti-angiogenic signals in wounds, and that the balance of these signals regulates the early angiogenic response during granulation tissue formation, and its resolution as the scar matures.

12.5 Wound Macrophages and the Alternatively Activated Phenotype

The capacity of macrophages to exhibit a variety of phenotypic profiles in response to different stimuli has been used for their classification into distinct functional categories (Stout and Suttles 2004). In its most simplified version, current paradigm holds that macrophages polarize either to a cytotoxic and microbicidal phenotype after stimulation with IFN-γ and lipopolysaccharide (the classically activated or M1 macrophage), or to an alternatively activated (M2) phenotype, associated with parasitic infections and allergic reactions (Gordon 2003).[1] Because these extreme phenotypes are not expressed in pure form in vivo, recent publications have embraced the view that macrophage activation is best described by a continuous spectrum of phenotypic characteristics (Mosser and Edwards 2008). The concept that wound macrophages express the alternatively activated phenotype has, however, been retained (Mosser and Edwards 2008; Lucas et al. 2010). Furthermore, it has been proposed that alternatively activated macrophages be re-classified as "wound healing macrophages" (Mosser and Edwards 2008). This recommendation is not supported by studies of macrophages directly isolated from wounds. Findings from this and other laboratories in sterile murine wounds demonstrated that wound macrophages exhibit a mixed and temporally varying phenotype with traits of both classically and alternatively activated cells, and that neither IL-4 (Daley et al. 2010; Mirza and Koh 2011), nor IL-13 (Daley et al. 2010), the proposed determinants of the "wound-healing alternatively activated" macrophage phenotype, are detectable in murine deep tissue wounds.

The expression of the enzyme arginase by alternatively activated (Gordon 2003) or "wound healing" (Mosser and Edwards 2008) macrophages has been singled out as central to their putative role in tissue repair. Arginases catalyze the degradation of arginine to ornithine and urea. Ornithine can, in turn, be metabolized to proline, an amino acid abundant in collagens. The demonstration of extracellular arginase activity in rat wounds led this laboratory to propose that ornithine-derived proline could promote or facilitate collagen synthesis by increasing its availability to fibroblasts and myofibroblasts (Albina et al. 1990). There is, however, no direct evidence that native proline is limiting to collagen synthesis in wounds, and thus that ornithine-derived proline is essential to repair. In fact, rat wound fibroblasts preferentially incorporate pre-formed, rather than ornithine-derived, proline into collagen (Albina et al. 1993). Moreover, native proline suppresses the use of

[1] Alternatively activated macrophages were first described by Gordon in 2003 as those arising from stimulation with IL-4 or IL-13 (Gordon 2003). Since then, at least three subtypes of M2 macrophages have been proposed to exist: M2a, which are those originally described by Gordon; M2b emerging from cells treated with immune complexes and TLR agonists; and M2c resulting from activation via glucocorticoids and IL-10 (Mantovani et al. 2004). Other investigators (Mosser and Edwards 2008) have also criticized the promiscuous use of the M2 designation to group a variety of activation states with distinct biologic and functional characteristics.

ornithine-derived proline during collagen synthesis by fibroblasts. Providing further evidence against arginase as a necessary regulator of collagen synthesis, a paradoxical increase in fibrosis following infection with *S. mansoni* was described in animals with macrophage-specific deletion of arginase I (Pesce et al. 2009).

Most importantly, arginase I is not expressed in human monocytes or macrophages, even after polarization towards "classical" or "alternatively" activated phenotypes (Munder et al. 2005; Raes et al. 2005). Contrasting the macrophage-restricted expression of arginase I in rodent wounds (Albina et al. 1990), arginase has been found in neutrophils in acute human wounds, and in fibroblasts in chronic wounds and ulcers, but not in macrophages (Debats et al. 2009; Jude et al. 1999). Arginase I is also expressed in human, but not in rodent, erythrocytes (Spector et al. 1985), and lysed red blood cells contribute arginase to human wounds. In this connection, arginase activity is highest in post-mastectomy wound fluids within 24 h of surgery and correlates with the hemoglobin concentration in the fluids (unpublished observations). The potential role of mammalian red blood cell arginase I in early inflammation and repair has not been examined. The expression of arginase by wound macrophages is, thus, species restricted and most likely not essential for normal collagen deposition in wounds.

It has also been proposed that another enzyme of arginine metabolism in macrophages, the inducible form of nitric oxide synthase (iNOS), is relevant to repair (Efron et al. 2000). The authors' laboratory first reported the presence of iNOS in rat wound macrophages (Albina et al. 1990; Reichner et al. 1999), and its absence in sterile murine wounds (Daley et al. 2010). The enzyme is, however, found in macrophages from murine wounds colonized with bacteria (Mahoney et al. 2002). Despite the time elapsed since the discovery of the nitric oxide synthases, the expression of iNOS in human macrophages remains controversial (Albina 1995; Weinberg 1998). There is, in addition, no evidence that iNOS is expressed by human macrophages in healing wounds.

Because of the aforementioned results, it is probably reasonable to avoid the M1 (classically activated) vs. M2 (alternatively activated) macrophage nomenclature, at least as it applies to the phenotype of the wound macrophages.

12.6 Origin of Wound Macrophages

Tissue injury is followed by an immediate inflammatory response that involves the recruitment of circulating polymorphonuclear leukocytes and monocytes to the wound. Monocytes arriving at the wound rapidly acquire certain macrophage phenotypic traits [i.e., mannose receptor-1 (CD206) (Daley et al. 2010), CD14 (unpublished observation)], while retaining others present in circulating monocytes [i.e., Ly6C, CCR2, and Dectin-1 (Daley et al. 2010)].

At least two distinct monocyte sub-populations have been identified in mice based on the expression of Ly6C and chemokine receptors CCR2 and CX3CR1 (Geissmann et al. 2003; Auffray et al. 2009). Ly6C provides a useful marker to

distinguish different populations of murine monocytes and macrophages, although its function in these cells remains unknown. Ly6C belongs to a family of cell surface phosphatidylinositol-anchored glycoproteins expressed to varying degrees in leukocytes and some endothelial cells (Gumley et al. 1995). No in vivo ligands for Ly6 family members have been identified. Because these molecules lack transmembrane or intracellular domains, a variety of transduction mechanisms for Ly6 have been proposed, including binding to intracellular Src tyrosine kinases through cytoskeletal proteins in cells of monocytic lineage (Gumley et al. 1995; Jaakkola et al. 2003). Antibody cross-linking of Ly6C in $CD8^+$ lymphocytes results in integrin activation and enhances adherence to endothelia (Jaakkola et al. 2003). A similar function in monocytes could regulate their trans-vascular migration into wounds, but this possibility has not been reported.

$Ly6C^{high}CCR2^{high}CX3CR1^{low}$ monocytes migrate into sites of inflammation, including wounds, during the early phase of the response to injury (Geissmann et al. 2003; Nahrendorf et al. 2007; Daley et al. 2010). The role of CCR2 expressed by $Ly6C^{high}$ monocytes in regulating their release from the bone marrow and their migration into wounds has been convincingly demonstrated (Geissmann et al. 2003; Daley et al. 2010). The inactivation of CCR2 by either genetic or pharmacologic means suppresses macrophage infiltration and the development of hepatic, renal, and pulmonary fibrosis in a variety of murine models (Seki et al. 2009; Li et al. 2008; Gharaee-Kermani et al. 2003). However, while the recruitment of $Ly6C^{high}$ monocytes into wounds of CCR2 KO mice is markedly reduced, the rate of closure of the wounds is not affected in the CCR2-deficient animals (Willenborg et al. 2012). Thus, although strong evidence supports the roles of CCR2/CCL2 in the recruitment of monocytes into wounds and other inflammatory lesions (Ishida et al. 2008; Dipietro et al. 2001), it appears that a more complex signal network is required for the normal migration of monocytes into wounds. In this regard, an antiserum against CCL3 (macrophage inflammatory protein 1α/MIP-1α, a chemokine that signals through chemokine receptors CCR1, 3, 5, and 9) decreased macrophage accumulation in wounds (DiPietro et al. 1998). In addition, a variety of genetic manipulations, including deletions of tumor necrosis factor-α receptor p55, β-1,4 galactosyltransferase, P/E selectin, or ICAM-1, result in reduced recruitment of macrophages to wounds and, with the exception of the TNFRp55 knockouts, in impaired wound healing [reviewed in (Eming et al. 2007)].

The role of the second population of circulating murine monocytes, defined as $Ly6C^{low}CCR2^{low}CX3CR1^{high}$, in the process of repair is less clear. This population was originally thought to be the precursor of tissue resident macrophages and shown to have little migratory activity towards sites of sterile inflammation (Geissmann et al. 2003). Whereas $CX3CR1^{int/high}$ macrophages are detected in increasing numbers in maturing murine wounds, their origin remains unsettled. Nahrendorf et al. (2007) proposed the sequential recruitment of $Ly6C^{high}CCR2^{high}CX3CR1^{low}$ and $Ly6C^{low}CCR2^{low}CX3CR1^{high}$ monocytes into areas of experimental myocardial infarction in mice. This time-dependent recruitment of monocyte subsets has not been found in other models of tissue injury. In the authors' laboratory, experiments using multiple in vivo approaches to track circulating monocytes demonstrated that

Ly6C^{low}CCR2^{low}CX3CR1^{high} monocytes fail to migrate into the wound.[2] Rather, the increasing number of Ly6C^{low}CCR2^{low}CX3CR1$^{int/high}$ macrophages found in the wound over time appears to derive from the in situ maturation of monocyte-derived Ly6C^{high} cells (unpublished observations). In a similar vein, Ly6C^{low}CX3CR1^{high} macrophages found in muscle injured through the injection of the myotoxin notexin were proposed to originate from the in situ maturation of Ly6C^{high} infiltrating monocyte-macrophages (Arnold et al. 2007).

Regardless of their origin, Ly6C^{high} and ^{low} macrophages in wounds differ by more than their surface marker expression. Early Ly6C^{high} wound macrophages express a mixed phenotype biased towards pro-inflammation, as witnessed by their abundant production of TNF-α and IL-6 (Daley et al. 2010; Brancato and Albina 2011). In contrast, late Ly6C^{low} wound macrophages release more TGF-β and VEGF than their earlier counterpoints, are efficient in presenting antigen to T cells, and exhibit an incomplete repertoire of dendritic cell markers (CD11c^{high}MHC-II^{high}CD80^{high}CD86^{high}CD205$^{low/-}$CD8a$^{low/-}$CCR7^{-}) (unpublished observations).

As just discussed, the role of CX3CR1 and its ligand CX3CL1 in recruiting monocytes into wounds remains unclear. It has been shown that CX3CR1/CX3CL1 convey survival signals to cells (Landsman et al. 2009; White and Greaves 2012). CX3CR1-deficient mouse monocytes contain less Bcl-2 and exhibit a reduced lifespan, and the overexpression of hBcl-2 restores the number of CX3CR1^{high} monocytes in the blood (Landsman et al. 2009). The role of CX3CR1 in prolonging monocyte/macrophage lifespan, rather than in recruiting Ly6C^{low} monocytes, has been proposed to explain the impaired healing of experimental myocardial infarctions reported by Nahrendorf et al. (2007) in CX3CR1-deficient animals (Gautier et al. 2009). The pathway through which CX3CR1 activation reduces cell death is not clear. Fractalkine (CX3CL1) induces calcium transients and G-protein/PI3K/Akt activation in cells overexpressing human or rat CX3CR1, but no PI3K/Akt signaling is detected in identical cells expressing the murine receptor. This difference was found to stem from a single amino acid mismatch in the receptor sequence among species (Davis and Harrison 2006). It may well be that the mechanism through which CX3CR1 protects monocytes/macrophages from apoptotic death differs among species or is independent of PI3K/Akt. Notwithstanding, CX3CR1 is expressed in murine skin wounds by infiltrating macrophages, myofibroblasts, and endothelial cells; and its ligand CX3CL1 is found in wound macrophages and endothelial cells. Cutaneous wound healing is retarded in CX3CR1 knockout animals, where wounds exhibit reduced number of macrophages and myofibroblasts, and decreased levels of TGF-β1 and VEGF (Ishida et al. 2008).

[2] Most results from the authors' laboratory discussed in this manuscript were obtained using the subcutaneously implanted polyvinyl alcohol sponge wound model in rodents (Albina et al. 1989; Mateo et al. 1994). The inflammatory, proliferative, and remodeling phases of wound healing are recapitulated in this model that, in addition, allows for the recovery and immediate study of specific wound cells, including macrophages, over time.

The relative roles of monocyte-derived versus resident tissue macrophages (such as osteoclasts, pulmonary alveolar macrophages, microglia, tissue histiocytes, etc.) in the repair of specific tissues and organs have not been completely defined. Recent evidence has shown the dual embryonic origin of myeloid cells of the monocyte/macrophage lineage in mice with most, if not all, resident tissue macrophages originating in the yolk sac of the developing embryo rather than from stem cells in the hematogenic endothelium or the fetal liver (Schulz et al. 2012). Given that resident tissue macrophages in soft tissues share markers with the $Ly6C^{low}$ macrophages found in maturing wounds, it is likely that at least some wound macrophages derive from resident macrophages rather than from the local maturation of $Ly6C^{high}$ cells originally migrating from the circulation. In this regard, it was recently reported that 30 % of macrophages in one-day-old skin wounds in irradiated/reconstituted mice derive from local tissue macrophages (Rodero et al. 2013). The participation of resident tissue macrophages in the process of repair may also explain the finding of proliferation markers in $Ly6C^{low}$ macrophages in muscle wounds (Arnold et al. 2007), because tissue macrophages are thought to retain proliferative activity (Liddiard et al. 2011).

There is additional evidence for cooperation between blood-derived and tissue macrophages in tissue repair. For example, resident Kupffer cells participate in the fibrotic response to a variety of hepatic insults by secreting cytokines and growth factors and by activating hepatic stellate cells, but the extent of fibrosis is markedly reduced in CCR2 knockouts unable to recruit blood monocytes into the injured liver (Seki et al. 2009).

Distinct populations of monocytes have also been described in humans, where they are distinguished by the expression of CD16 (reviewed in Auffray et al. 2009). $CD16^{+}$ cells, which normally constitute 5–15 % of circulating monocytes, express high levels of CX3CR1 and may be the ortholog of $Ly6C^{low}CX3CR1^{high}$ murine monocytes. The normally more abundant $CD16^{-}$ monocytes are $CX3CR1^{low}$, and resemble $Ly6C^{high}$ murine monocytes in their expression of CCR2. A third population of human monocytes expressing both CD14 and CD16 has been shown to comprise at least two sub-populations with distinct capacities to release TNF-α and IL-1 in response to lipopolysaccharide. The function of these $CD14^{+}CD16^{+}$ cells remains unclear, but their frequency in the circulation increases in septic diseases (Auffray et al. 2009). Whether human monocytes specifically parallel their murine counterparts in their capacity to migrate into wounds and have differential secretory and functional profiles at a site of injury remains to be investigated.

12.7 Fate of Wound Macrophages

Macrophages disappear from wounds during the resolution of the inflammatory phase of repair. The concept that macrophages are resistant to apoptotic stimuli and migrate out of inflammatory spaces to regional draining lymph nodes has been based on findings in peritonitis in rodents (Bellingan et al. 1996) and after the instillation of fluorescent beads into the lungs of dogs (Harmsen et al. 1985). More recent reports using a murine model of aortic arteriosclerosis failed to document

macrophage emigration through lymphatics and attributed the time-dependent reduction in the number of plaque-associated macrophages mainly to decreased monocyte recruitment (Potteaux et al. 2011). In a similar vein, work in the authors' laboratory failed to observe the migration of fluorescent latex bead-labeled wound macrophages to regional lymph nodes (unpublished observation). While migration or altered recruitment may explain the fate of wound macrophages, a least some of these cells die at sites of injury. This was evidenced by the accumulation of intracellular macrophage molecules in the wound extracellular fluid [most notably macrophage arginase I in rat wounds (Albina et al. 1990)].

It has been proposed that macrophages can transdifferentiate into fibroblasts and myofibroblasts in a variety of inflammatory pathologies and contribute to the deposition of collagen and other extracellular matrix components (Mooney et al. 2010; Ninomiya et al. 2006; Jabs et al. 2005; Bertrand et al. 1992). It remains unclear whether mature macrophages can undergo true lineage reprogramming into fibroblasts and myofibroblasts, or whether the co-expression of myeloid and mesenchymal markers in some inflammatory cells is the result of the differentiation of common precursors. In this connection, Bucala et al. first described the fibrocyte, a bone marrow derived circulating cell expressing CD34, CD45, and CD11b, and capable of producing extracellular matrix proteins and metalloproteinases (Bucala et al. 1994; Blakaj and Bucala 2012). Further work determined that human $CD14^{+}CD16^{-}$ monocytes can differentiate into fibrocytes under the influence of TGF-β or following direct contact with T cells (Bellini and Mattoli 2007). Fibrocytes are immature mesenchymal cells producing relatively small amounts of collagenous proteins and substantial quantities of matrix metalloproteinase-9 (Schmidt et al. 2003; Hartlapp et al. 2001). Fibrocytes can further differentiate into fibroblasts and myofibroblasts when stimulated with TGF-β and endothelin-1 (Schmidt et al. 2003). The population that emerges from this differentiation stage produces more collagen and fibronectin than the originating fibrocyte and expresses the myofibroblast marker α-smooth muscle actin (Schmidt et al. 2003). The relevance of fibrocytes in a variety of fibrotic disorders has been recently reviewed in (Bellini and Mattoli 2007).

The potential conversion of macrophages to reparative fibroblasts and myofibroblasts would provide an attractive alternative pathway for the disappearance of macrophages from wounds. Evidence has been reported, however, against a significant contribution of bone marrow derived cells to scar formation in skin wounds (Barisic-Dujmovic et al. 2010; Higashiyama et al. 2011). It follows that the relative contribution of mesenchymal transdifferentiation to the fate of the wound macrophage remains to be fully examined.

12.8 Role of Apoptotic Cells in the Regulation of the Wound Macrophage Phenotype

Macrophages are known to respond to signals received from soluble mediators and from components of the extracellular matrix. These signals undoubtedly participate in the regulation of the wound macrophage phenotype.

The authors' laboratory has been particularly interested in the role of neutrophils in modulating the wound macrophage phenotype. In this regard, neutrophils are the first blood-borne nucleated cells to migrate into injured tissues. They undergo apoptosis in the wound and are recognized and ingested by macrophages (Metchnikoff 1892; Meszaros et al. 1999). Rat wound macrophages actively induce apoptosis in neutrophils (Meszaros et al. 2000). The induction of neutrophil apoptosis requires the expression of membrane-bound TNF-α (but not soluble TNF-α), β_3 integrins, and CD36 by the effector macrophages. Neither resident nor *P. acnes*-elicited peritoneal macrophages induced apoptosis in neutrophils, indicating this ability to be particular to wound macrophages (Meszaros et al. 2000).

The phagocytosis of apoptotic neutrophils or other cells has been shown to induce an anti-inflammatory phenotype in macrophages. This phenotype includes the release of TGF-β and PGE_2, and a reduced ability to produce pro-inflammatory mediators such as TNF-α after lipopolysaccharide stimulation (Savill and Fadok 2000). Because $Ly6C^{high}$ wound macrophages produce TNF-α, and $Ly6C^{low}$ murine monocytes are more efficient than $Ly6C^{high}$ cells in the ingestion of apoptotic cells (Peng et al. 2009), it appears reasonable to propose that $Ly6C^{high}$ cells induce apoptosis in wound neutrophils while $Ly6C^{low}$ cells remove post-apoptotic debris from the wound space. Recent findings in the authors' laboratory support this hypothesis. $Ly6C^{low}$ macrophages in the maturing wound were found to express MERTK (unpublished observation). MERTK and other members of the TAM family of receptor protein tyrosine kinases (AXL and TYRO3) have been shown to play multiple roles in immune modulation, and specifically to be involved in the phagocytosis of apoptotic cells (Scott et al. 2001; Linger et al. 2008). The phagocytosis of apoptotic bodies (Linger et al. 2008) or of cell-derived microvesicles, including human neutrophil-derived ectosomes (Eken et al. 2010), by macrophages suppresses the production of pro-inflammatory cytokines via the inhibition of p65 phosphorylation and the translocation of NF-κB, and promotes the release of VEGF (Park et al. 2012). Whether the TAM receptor-mediated phagocytosis of apoptotic cells in wounds fully regulates the phenotype of macrophages during wound repair remains to be determined. In this connection, while the secretory phenotype of the murine $Ly6C^{low}MERTK^{high}$ wound macrophage is characterized by the simultaneous release of both VEGF and TGF-β, it has been reported that the production of the latter is not regulated via activation of MERTK (Park et al. 2012). It appears, thus, that additional signals independent of the phagocytosis of wound neutrophils are required for the acquisition of the full secretory profile of wound macrophages. In this regard, wound neutrophils release lipid-soluble low molecular weight factors (including PGE_2) that suppress the production of pro-inflammatory mediators by macrophages (Daley et al. 2005). The picture that emerges from these studies is that wound neutrophils, by serving as phagocytic targets and releasing soluble "pacifying" mediators, actively modulate the wound macrophage phenotype.

The impaired wound healing associated with diabetes has been correlated with persistent inflammation (Wetzler et al. 2000) and a failure of wound macrophages to transition from an inflammatory to a repair phenotype (Mirza and Koh 2011).

Providing a possible mechanistic connection between impaired healing in diabetes and macrophage/neutrophils in the wound, it has been proposed that defective efferocytosis of apoptotic neutrophils underlies the persistent inflammatory phenotype of macrophages in diabetes and accounts for the accumulation of apoptotic cells in wounds in diabetic mice and humans (Khanna et al. 2010; Mirza and Koh 2011).

While it has been demonstrated that efferocytosis by macrophages modulates the cells' phenotype towards anti-inflammation, this response appears to require a certain balance between the number of target apoptotic cells and effector macrophages. The induction of excess apoptosis in wounds via anti-CD95 antibodies resulted in the accumulation of pro-inflammatory mediators, reductions in wound TGF-β and IL-10 contents, and impaired wound closure (Khanna et al. 2010). In addition, the injection of apoptotic cells into the peritoneal cavity of mice elicited an acute inflammatory response characterized by the MIP-2/CXCR2-mediated recruitment of additional neutrophils (Misawa et al. 2001; Iyoda and Kobayashi 2004). A potential explanation for the aforementioned findings may lie in the engagement of alternative phagocytic receptors capable of inducing inflammatory cytokine production in macrophages by an overwhelming number of phagocytic targets.

Studies in CD18 knockout mice, which model for leukocyte-adhesion deficiency syndrome 1, a disorder characterized by recurrent infections and impaired wound healing in humans, have provided further information regarding macrophage/neutrophil interactions in wounds (Peters et al. 2005). Skin wounds in these animals failed to recruit neutrophils, assembled a normal complement of macrophages, and exhibited severe retardation of closure by contraction. These alterations occurred in the context of increased pro-inflammatory cytokine accumulation, reduced active TGF-β1 and its type II receptor, and a remarkable scarcity of myofibroblasts. It was proposed from these findings that the lack of apoptotic neutrophils in the wounds of knockout animals deprived macrophages of phagocytic substrate resulting in reduced production of TGF-β1, a main regulator of myofibroblast differentiation (Peters et al. 2005).

12.9 Future Directions

Evidence discussed so far argues against the concept that wound macrophages express one "repair" phenotype, identical to that of the "alternatively activated" macrophage. A new perspective into the nature of wound macrophages has emerged from the discovery that wound macrophages have a dynamic phenotype, and express temporally restricted phenotypes that instruct other wound cells through the different phases of repair.

It is likely that a number of variables known to affect wound healing, including wound infection, co-morbidities (i.e., diabetes, malnutrition, cancer, etc.), and even the age of the wounded subject, may do so by impacting on the wound macrophage. Further work is needed to examine wound macrophages in these and other physiologic and pathologic conditions.

The phenotype of human wound macrophages has been under-investigated. The substantial differences reported by this and other laboratories between human and murine macrophage phenotypes, and even between wound macrophages of mice and rats, suggest that findings in rodents may have limited parallels in human wound macrophages. The development of models and methods that allow for the study of wound cell biology in human wounds is highly desirable.

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Chapter 13
Macrophages and the Entrance of Resolution Phase Lipid Mediators

Charles N. Serhan, Nan Chiang, and Jesmond Dalli

Abbreviations

17*R*-HDHA	17*R*-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid
AT	Aspirin-triggered
AT-RvD3	4*S*,11*R*,17*R*-trihydroxydocosa-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-hexaenoic acid
LTB_4	Leukotriene B_4; 5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid
LXA_4	Lipoxin A_4; 5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid
LXB_4	Lipoxin B_4; 5*S*,14*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid
MaR1	Maresin 1; 7*R*,14*S*-dihydroxy-docosa-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexaenoic acid
MΦ	Macrophages
PD1/NPD1	Protectin D1/neuroprotectin D1; 10*R*,17*S*-dihydroxy-4Z,7*Z*,11*E*,13*E*,15*Z*,19*Z*-docosahexaenoic acid
PGE_2	Prostaglandin E_2; 9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid
Rv	Resolvin

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RvD1	Resolvin D1; 7*S*, 8*R*,17*S*-trihydroxy-4*Z*, 9*E*, 11*E*, 13*Z*, 15*E*, 19*Z*-docosahexaenoic acid
RvD2	Resolvin D2; 7*S*, 16*R*, 17*S*-trihydroxy-4*Z*, 8*E*, 10*Z*, 12*E*, 14*E*,19*Z*-docosahexaenoic acid
RvD3	Resolvin D3; 4*S*,11*R*,17*S*-trihydroxydocosa-5*Z*,7*E*,9*E*,13*Z*,15*E*, 19*Z*-hexaenoic acid
RvD5	Resolvin D5; 7*S*,17*S*-dihydroxy-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-docosa-hexaenoic acid
RvE1	Resolvin E1; 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapen-taenoic acid
RvE2	Resolvin E2; 5*S*,18*R*-trihydroxy-6*E*,8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid
SPM	Specialized pro-resolving mediators
TRP	Transient receptor potential

13.1 Introducing Resolvins, Protectins, and Maresins: The SPM

All cells in multi-cellular organisms require chemical gradients of signals that instruct each to move and/or stop as needed. In the acute inflammatory response many chemical signals are produced. Some are from exogenous microbial origins while others are biosynthesized by the host in response to tissue injury and invasion (Majno 1982). Among the chemical signals at the site of an acute inflammatory response (Buckley et al. 2013), those that originate from host essential fatty acids are of particular interest because of their nutritional regulation of the response (Zhang and Spite 2012) and the potential to design small molecule mimetics of these molecules (Serhan 2004). Those produced from arachidonic acid including prostaglandins and leukotriene B_4 are involved in the initiating steps that permit white blood cells to leave the post-capillary venules, i.e., diapedesis (Serhan et al. 2010). This laboratory focused on mechanisms involved in endogenous anti-inflammation and its resolution (Serhan 2004; Serhan et al. 2000; Levy et al. 2002). Using a systems approach, with LC-MS-MS-based lipidomics, in vivo animal models, exudate cell trafficking and functional assessment with isolated human cells, we identified novel bioactive products produced in the resolution phase of acute sterile inflammation (illustrated in Fig. 13.1) that activate pro-resolving mechanisms (Serhan et al. 2000, 2002; Hong et al. 2003). Focusing on self-limited resolving exudates also permitted a direct assessment of the host's responses that enables the return to homeostasis. A key bioassay that proved critical in the initial studies focused on human polymorphonuclear neutrophils (PMN) transmigration across endothelial cells and epithelial cells (Serhan et al. 2000). Attention was focused here because neutrophils are among the first responders to injury and microbial invasion. Our hypothesis that endogenous chemical mediators are produced via cell–cell interactions within inflammatory exudates (i.e., pus) that control the size,

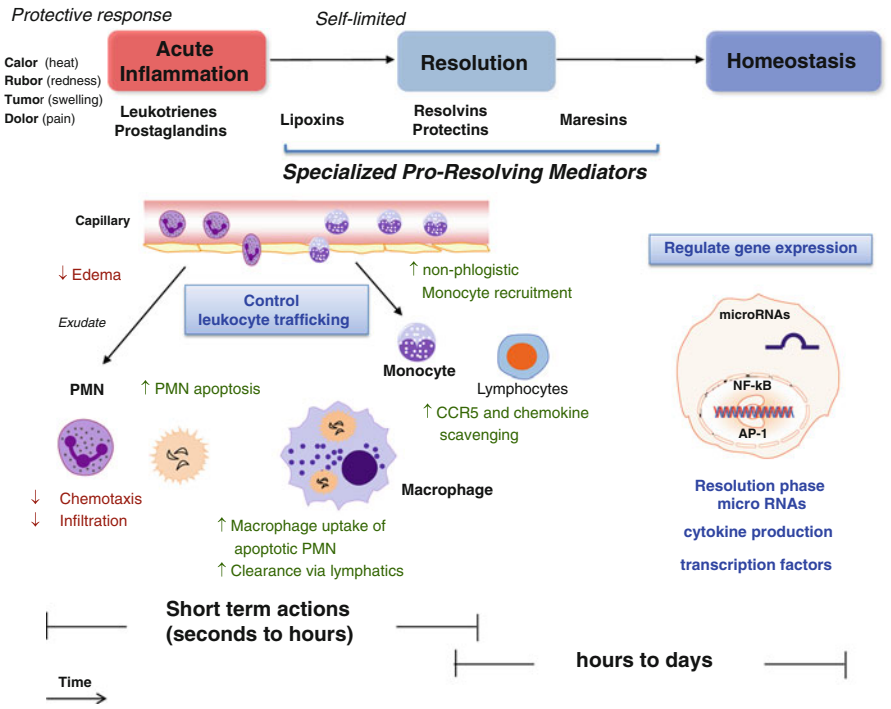

Fig. 13.1 SPM are resolution agonists displaying short- and long-term actions promoting tissue homeostasis. SPM are generated during inflammation-resolution and control the early events in acute inflammation such as edema formation, leukocyte trafficking, and functions; SPM reduce further PMN infiltration, and stimulate macrophage uptake of apoptotic PMN as well as their clearance via lymphatics. SPM also possess long-term actions, such as increasing chemokine scavenging by lymphocytes, and regulation of transcription factors, cytokine production, and select micro RNAs as reported recently. Summation of these unique resolution characteristics of SPM at multi-levels accelerates resolution of inflammation

magnitude, and duration of the inflammatory event proved to be the case and is relevant to human translation, as anti-PMN therapy (Takano et al. 1998) to limit tissue damage and uncontrolled inflammation has increasing appeal. The historical milestones in resolution of inflammation from observation to active resolution to new therapeutics first in humans are listed in Table 13.1. In ancient medical texts of the eleventh and twelfth centuries, the notion of treating inflammation with resolvents as mollificants to resolve disease was already present. Yet, this concept was apparently lost until the structures of endogenous mediators that stimulate resolution were elucidated (Serhan et al. 2002). From this line of investigation the resolvins, protectins, and maresins were uncovered. Within exudates resolving to homeostasis, the fundamental cellular processes proved predictive of the actions of specialized pro-resolving mediators (SPM) in complex disease models, because cessation of PMN entry into tissue and the removal of dead PMN as well as anti-PMN regulators are central to many organs and disease pathologies.

Table 13.1 Milestones to resolution of inflammation and tissue regeneration links to novel therapeutics[a]

Year	Discovery	References
Eleventh century; translated to the West in the twelfth century	Resolvent mollificants for treating "inflammatory foci" (The Canon of Medicine)	Avicenna, adapted by Laleh Bakhtiar (1999)
1979	Inflammatory exudate resolved vs. exudate organized	Robbins and Cotran (1979)
1988	Cellular events in resolution of inflammation Macrophage clearing of dead PMN and debris	Savill et al. (1989a, b) and Haslett and Henson (1988)
1994	Complete resolution as an outcome of acute inflammation; chronic inflammation as a failure to resolve	Cotran et al. (1994) and Serhan (1994)
1996	Differential signals for chemical mediators Lipoxins are a stop signal for PMN recruitment and nonphlogistic stimuli for monocytes	Maddox and Serhan (1996)
1999	COX-2 may have anti-inflammatory properties	Gilroy et al. (1999)
1999	Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes	Liu et al. (1999)
2000	Lipoxins stimulate macrophage phagocytosis of apoptotic PMN	Godson et al. (2000), Perretti et al. (2002), and Maderna et al. (2005, 2010)
2000	Discovery of resolvin E1 and the link to ω-3 PUFA Resolution is a biosynthetically active process	Serhan et al. (2000)
2001	Mediator eicosanoid class switching from prostaglandins and leukotrienes to lipoxins in resolution	Levy et al. (2001)
2002	Discovery of the resolvins, protectins, and their aspirin-triggered forms	Serhan et al. (2002)
2002	Peptide mediators in resolution; annexin activation of ALX/FPR-2 receptor and enhanced macrophage phagocytosis	Perretti et al. (2002) and Maderna et al. (2005, 2010)
2006	Efferocytosis of apoptotic PMN; removal of macrophages; failure to chronicity	Vandivier et al. (2006)
2007	Consensus report on resolution of inflammation: definitions and terms	Serhan et al. (2007)
2009	Report of the first patients treated with a resolvin, an agonist to stimulate resolution and treat inflammation	Brooks (2009)
2011	Resolvin E1 controls HSV viral infection in keratitis Protectin D1 controls influenza virus replication	Rajasagi et al. (2011) and Morita et al. (2013)
2012	First clinical trial with LXA_4 stable analog, reducing severity of eczema	Wu et al. (2013)
2012	Resolvins and protectins control bacterial infection and enhance antibiotic effectiveness	Chiang et al. (2012)

[a]Updated from Serhan (2011)

Of particular interest, we learned that n-3 essential fatty acids EPA and DHA serve as substrates within inflammatory resolving exudates for the biosynthesis of potent anti-inflammatory pro-resolving endogenous mediators (Serhan et al. 2002; Hong et al. 2003). The identification of omega-3 fatty acids as nutrients that activate pro-resolving mechanisms in inflammation opens new areas of investigation since uncontrolled inflammation is now widely appreciated to be the basis of many widely occurring diseases (Fig. 13.1). It is worth noting that a large body of literature addressing the anti-inflammatory impact of EPA and DHA is present (for a recent review, see Calder 2013); yet, the molecular mechanism(s) by which these essential fatty acids could exert their anti-inflammatory actions remained the subject of discussion. DHA and EPA have many known critical functions in mammalian biology. Neither EPA nor DHA is produced by humans to any great extent and therefore must be taken in the diet (Calder 2013). One area where the function of DHA and its mechanism is clear is in the role of DHA in the retina and vision. As recently discussed, DHA is an ancient molecule that has arisen, with functional roles in brain and eye imposed via evolutionary pressure (Crawford et al. 2013). Hence, uncovering novel chemical mediators that are biosynthesized during self-limited inflammatory responses with inflammatory exudates in murine systems with functions on individual mammalian and human leukocytes has far-reaching implications (Table 13.2). For further mechanistic details on these SPM (resolvins, protectins, and maresins), interested readers are directed to recent reviews covering SPM biosynthesis (Bannenberg and Serhan 2010), actions, and total organic synthesis (Serhan and Petasis 2011). In this chapter, we review key initial observations that permitted a rapid expansion and confirmation from many laboratories of the SPM biosynthesis and activities on phagocytes and in animal models of disease.

13.1.1 What Makes Pro-resolving Different from Anti-inflammation? Defining Novel Immunoresolvents

Each SPM native structure possesses potent pro-resolving actions that include limiting or cessation of neutrophil PMN tissue infiltration, counter-regulation of chemokines (Serhan et al. 2002; Hong et al. 2003) and cytokines, reduction in pain (Xu et al. 2010), and stimulation of macrophage-mediated actions, i.e., efferocytosis, bacterial, and debris clearance (Schwab et al. 2007; Chiang et al. 2012) (Table 13.3). Thus, SPM are agonists that act on both PMN and macrophages separately to stimulate resolution. Given this unique mechanism of pro-resolving actions, resolvins and SPM demonstrate potent actions in animal disease models (Tables 13.1 and 13.4). Like other autacoids, the actions of resolvins and SPM are stereochemically selective, reflecting their routes of biosynthesis and underlying their ability to activate receptors (G protein-coupled receptor; GPCR) that amplify and transduce their tissue response. The classic eicosanoids each carry well established

Table 13.2 SPM production in humans

SPM	Disease/tissues	Formation
Lipoxins & Aspirin-triggered lipoxins (ATL)	Colitis	Elevated mucosal LXA_4 in ulcerative colitis individuals in remission (Vong et al. 2012)
	Type 2 diabetes	Increased plasma ATL with intake of pioglitazone (Gutierrez et al. 2012)
	Rheumatoid arthritis	LXA_4 present in synovial fluid from rheumatoid arthritis patients (Giera et al. 2012)
	Localized aggressive periodontitis (LAP)	Less LXA_4 in LAP whole blood compared to healthy individuals (Fredman et al. 2011)
	Asthma	Higher urinary ATL levels in aspirin-tolerant asthma than in aspirin-intolerant asthma (Yamaguchi et al. 2011)
	Peripheral artery disease	Lower plasma levels of ATL in patients with symptomatic peripheral artery disease than in healthy volunteers (Ho et al. 2010)
	Adipose tissues	LXA_4 identified in human adipocytes from obese patients (Clària et al. 2012)
Resolvins	Synovial fluid	RvD5 present in synovial fluid from rheumatoid arthritis patients (Giera et al. 2012)
	Blood (healthy volunteers)	Plasma RvD1 and RvD2 identified with oral omega-3 supplementation (Mas et al. 2012)
	Adipose tissues	RvD1 and RvD2 identified in human adipocytes from obese patients (Clària et al. 2012)
	Human plasma	RvE1 identified in human plasma (~1 nM) (Psychogios et al. 2011)
	Multiple sclerosis	RvD1 was detected and upregulated in serum and cerebrospinal fluid in the highly active group (Pruss et al. 2013)
Protectin	Asthma	Decreased PD1 in eosinophils from patients with severe asthma compared to healthy individuals (Miyata et al. 2013)
	Embryonic stem cells	PD1 produced in embryonic stem cells (Yanes et al. 2010)
	Multiple sclerosis	NPD1 was detected in serum and cerebrospinal fluid in the highly active group (Pruss et al. 2013)
Maresins	Synovial fluid	MaR1 is identified in synovial fluid from rheumatoid arthritis patients (Giera et al. 2012)

stereoselectivity in their actions (Samuelsson 2012; Shimizu 2009). Hence, establishing the complete stereochemical assignment for each of the separate members of the resolvin, protectin, and maresin (SPM) families of structures shown in Figs. 13.2, 13.3, and 13.4 was key to confirming novel actions on leukocytes. Given their ability to stimulate resolution of inflammation without systemic immune suppression (see below and Chiang et al. 2012), we have come to recognize that SPM are immunoresolvents; by definition, they stimulate resolution (Serhan et al. 2012; Serhan 2011).

Table 13.3 Phagocytosis in vivo and in vitro: Role of SPM in host defense

	PMN	Macrophages	In vivo
LXA_4 ATL		Apop PMN (Godson et al. 2000; Schwab et al. 2007) STZ (Schwab et al. 2007) Latex beads (Schwab et al. 2007) *E. coli* (Prescott and McKay 2011)	STZ (Schwab et al. 2007) Apop PMN (El Kebir et al. 2009) *E. coli* (El Kebir et al. 2009) Multimicrobial sepsis/CLP (Walker et al. 2011)
RvE1	*Candida albicans* (Haas-Stapleton et al. 2007)	Apop PMN (Schwab et al. 2007; Oh et al. 2011) STZ (Schwab et al. 2007; Ohira et al. 2010; Oh et al. 2011) Latex beads (Schwab et al. 2007) *E. coli* (Oh et al. 2011)	STZ (Schwab et al. 2007) HSV-1 (Rajasagi et al. 2011) *E. coli* (Seki et al. 2010; El Kebir et al. 2012)
18S-RvE1		Apop PMN (Oh et al. 2011) STZ (Oh et al. 2011) *E. coli* (Oh et al. 2011)	
RvE2		STZ (Oh et al. 2011)	
PD1	*E. coli* (Chiang et al. 2012)	STZ (Schwab et al. 2007) Apop PMN (Schwab et al. 2007) Latex beads (Schwab et al. 2007) *E. coli* (Chiang et al. 2012)	STZ (Schwab et al. 2007) Apop PMN (El Kebir et al. 2009) *E. coli* (Chiang et al. 2012)
RvD1	*E. coli* (Chiang et al. 2012)	STZ (Krishnamoorthy et al. 2010) Apop PMN (Krishnamoorthy et al. 2010) *E. coli* (Chiang et al. 2012)	*E. coli* (Chiang et al. 2012) Apop PMN (Hsiao et al. 2013)
AT-RvD1		*E. coli* (Palmer et al. 2011)	
RvD2	*E. coli* (Spite et al.)	STZ (Spite et al. 2009)	Multimicrobial sepsis/CLP (Spite et al. 2009)
RvD3 AT-RvD3		STZ (Dalli et al. 2013a) Apop PMN	
RvD5	*E. coli* (Chiang et al. 2012)	*E. coli* (Chiang et al. 2012)	*E. coli* (Chiang et al. 2012)
MaR1		STZ (Serhan et al. 2009) Apop PMN (Serhan et al. 2012)	

STZ, serum treated zymosan

13.2 Confirming the Structures and Stereochemical Assignments

It was essential to confirm the structure and novel potent actions of each of the resolvins and SPM. To this end, we devised a systematic approach to matching endogenous produced SPM to those prepared by total organic synthesis. This approach was deemed necessary because the SPMs are produced in only small quantities in vivo in the picogram to nanogram range and act locally and are inactivated (reviewed in Serhan and Petasis 2011). These transient small quantities

Table 13.4 Some recent new actions of SPM in vivo in disease models

Disease	SPM	Bioaction
Alzheimer's disease (AD)	RvD1	Stimulates phagocytosis of Aβ by AD macrophages (Mizwicki et al. 2013)
Burn wound	RvD2	Prevents secondary thrombosis and necrosis (Bohr et al. 2013)
Chronic pancreatitis	RvD1	Reverses allodynia (Quan-Xin et al. 2012)
Diabetic wounds	RvD1	Accelerates wound healing (Tang et al. 2013)
Dermatitis	RvE1	Ameliorates dermatitis (Kim et al. 2012)
Pulmonary inflammation	RvE1	Promotes apoptosis and accelerate resolution (Seki et al. 2010)
Peripheral nerve injury	RvE1	Inhibits neuropathic pain (Xu et al. 2013)
Obesity	RvD1, RvD2	Govern inflammatory tone (Clària et al. 2012)
Allergic airway response	RvD1, AT-RvD1, RvE1	Promote resolution (Rogerio et al. 2012; Haworth et al. 2011)
ALS	RvD1	Inhibits inflammation (Liu et al. 2012)
Acute lung injury	AT-RvD1	Reduces mucosal inflammation (Eickmeier et al. 2013)
Fibrosis	RvE1, RvD1	Inhibit kidney fibrosis (Qu et al. 2012)
Bacterial infection	RvD1, RvD5, PD1	Increase survival and lower antibiotic requirement (Chiang et al. 2012)
Peritonitis	RvD1	Limits PMN recruitment and accelerate resolution (Norling et al. 2012; Recchiuti et al. 2011)
Dry eye	RvE1 and analog	Protect from goblet cell loss (de Paiva et al. 2012); improves tear production (Li et al. 2010)
Tissue regeneration	RvE1, MaR1	Promote tissue regeneration in planaria (Serhan et al. 2012)
Pain	MaR1, RvD1, AT-RvD1, RvD2, RvE1	Control inflammatory pain (Serhan et al. 2012; Park et al. 2011; Bang et al. 2010, 2012; Xu et al. 2010)
Adipose tissue inflammation	RvD1	Elicits macrophage polarization and promote resolution (Titos et al. 2011)
Localized aggressive periodontitis	RvE1	Rescues impaired phagocytosis (Fredman et al. 2011)
Colitis	RvD1, RvD2, RvE1	Prevent colitis (Bento et al. 2011; Ishida et al. 2010)
TMJ inflammation	AT-RvD1	Limits PMN infiltration to CFA-inflamed TMJ (Norling et al. 2012)
Arthritis	AT-RvD1	Anti-hyperalgesic (Lima-Garcia et al. 2011)
Postoperative pain	RvD1	Prevents and reduces pain (Huang et al. 2011)
Endotoxin shock	RvD1	Suppresses septic mediators (Murakami et al. 2011)
HSV-keratitis	RvE1	Controls ocular inflammatory lesions (Rajasagi et al. 2011)
Allograft rejection	RvE1	Preserves organ function (Levy et al. 2011)
Heart Ischemia	RvE1	Protects heart against reperfusion injury (Keyes et al. 2010)
Bacterial pneumonia	RvE1	Protects mice from pneumonia (Seki et al. 2010)
Cigarette smoke-induced lung inflammation	RvD1	Promotes M2 macrophages and efferocytosis as well as accelerates resolution of lung inflammation (Hsiao et al. 2013)
Vascular inflammation (arterial angioplasty)	RvD1	Attenuates cell proliferation, leukocyte recruitment, and neointimal hyperplasia (Miyahara et al. 2013)

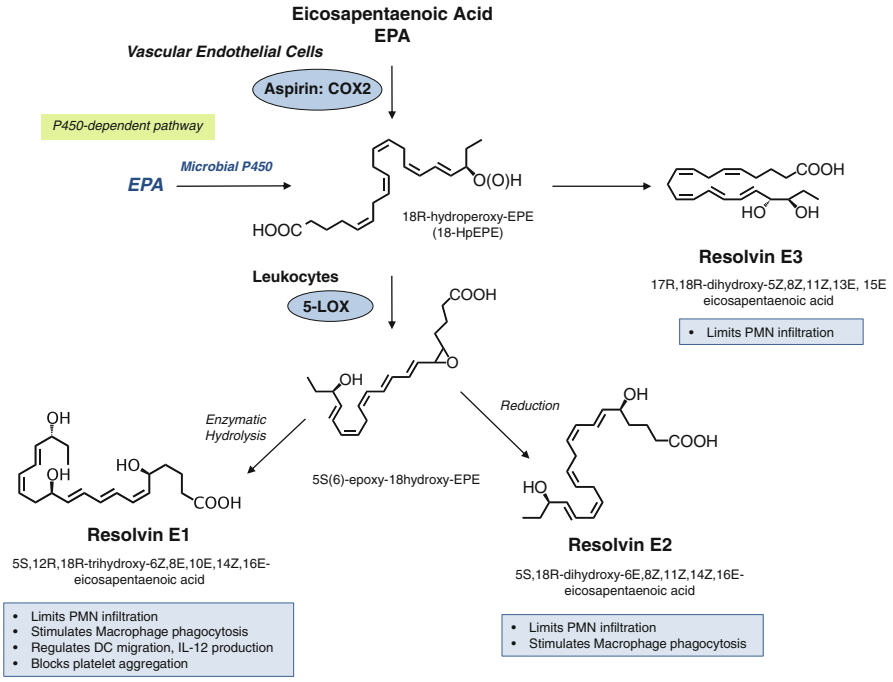

Fig. 13.2 EPA resolution metabolome—E-series Resolvin biosynthetic pathways. Biosynthetic scheme for E-series resolvins and their actions

precluded direct NMR analysis. The original identification of the D-series resolvins reported the structural elucidation of several distinct bioactive structures denoted resolvin D1 through resolvin D6 in resolving murine exudates, their biosynthesis by isolated human leukocytes (Fig. 13.3) and potent actions in vivo in murine as well as human acute inflammation (Table 13.4) (Serhan et al. 2002). Recently, the structure of resolvin D1 (RvD1), its aspirin-triggered 17*R*-epimer (Sun et al. 2007), RvD2 (Spite et al. 2009), aspirin-triggered (AT)-protectin D1 (PD1) (Serhan et al. 2011), and maresin 1 (MaR1) (Serhan et al. 2012) were assigned and several made available from Cayman Chemical. Recently, we establish the complete stereochemistry of the third member of the D-series RvD3 and its AT-RvD3 with materials prepared by stereocontrolled total organic synthesis (Dalli et al. 2013a). For example, using LC-MS/MS metabololipidomics we matched the physical properties of RvD3 (Fig. 13.3) with those of synthetic materials possessing the stereochemistry that proved to be 4*S*,11*R*,17*S*-trihydroxydocosa-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-hexaenoic acid and the AT-RvD3 matched synthetic product 4*S*,11*R*,17*R*-trihydroxydocosa-5*Z*,7*E*,9*E*,13*Z*,15*E*,19*Z*-hexaenoic acid. When administered in vivo both synthetic epimers demonstrated a potent reduction (40–50 %) in murine PMN recruitment to sites of inflammation at doses as low as 10 pg/mouse. Systemic administration of both RvD3 and AT-RvD3 significantly increased exudate IL-10 between 5 and 50 %; IL-6 was reduced ~50–65 %; and eicosanoids, i.e. LTB_4, ~85 % (Dalli et al. 2013a).

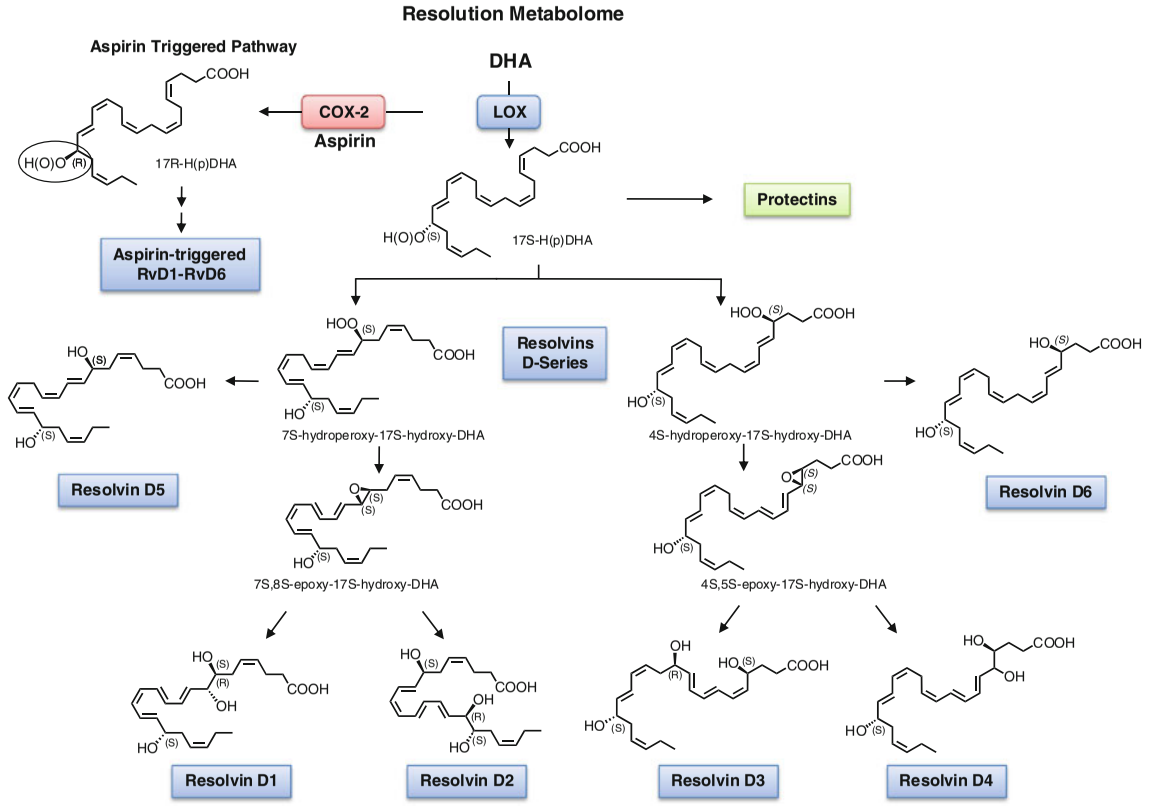

Fig. 13.3 DHA resolution metabolome—D-series Resolvin biosynthetic pathways. Biosynthetic scheme for D-series resolvins and their relation to protectins. Note that the complete stereochemistry of RvD1, RvD2, and RvD3 are established as shown. See Dalli et al. (2013a), Sun et al. (2007), and Spite et al. (2009) for further details

Local administration of either RvD3 or AT-RvD3 regulated the local release of pro-inflammatory cytokines/chemokines, including MCP-1 (~60 %) in this system. With human leukocytes, RvD3 and AT-RvD3 each potently regulates leukocyte functions enhancing peritoneal macrophage phagocytosis (20–60 %) and efferocytosis (20–40 %) in a dose dependent manner whilst reducing human neutrophil transendothelial migration in response to TNFα. These results establish the complete stereochemistry and confirmed the potent anti-inflammatory and pro-resolving actions of RvD3 and its aspirin-triggered epimer AT-RvD3. In addition, lipid mediator metabololipidomic profiling of self-resolving exudates also placed RvD3 uniquely within the time course of inflammation-resolution to vantage complete resolution.

13.3 Maresins: Macrophage Mediators in *Res*olving *In*flammation

Recently, we also established the stereochemical assignments for AT-PD1 (Serhan et al. 2011) and that of maresin 1 (MaR1) (Serhan et al. 2012). MaR1 produced by human macrophages (MΦ) from endogenous docosahexaenoic acid (DHA) matched the stereochemistry of synthetic 7*R*,14*S*-dihydroxydocosa-4Z,8*E*,10*E*,12*Z*,*16Z*,*19Z*-hexaenoic acid. MaR1 alcohols groups and Z/E geometry of conjugated double bonds were assigned using isomers prepared by total organic synthesis. MaR1's potent defining actions were confirmed with synthetic MaR1, i.e., limiting neutrophil (PMN) infiltration in murine peritonitis (ng/mouse range) as well as enhancing human macrophage uptake of apoptotic PMNs. At 1nM, MaR1 was slightly more potent than Resolvin D1 (RvD1) in stimulating human MΦ efferocytosis, an action not shared by leukotriene B_4. Importantly, MaR1 also accelerated surgical regeneration in planaria, increasing the rate of head reappearance. Upon injury of the planaria, MaR1 is biosynthesized from deuterium-labeled (d_5)-DHA. MaR1 dose-dependently inhibited TRPV1 currents in neurons, blocked capsaicin [100 nM]-induced inward currents $IC_{50} \approx 0.5$ nM, and reduced both inflammatory and chemotherapy-induced neuropathic pain in mice. Hence, MaR1 has potent actions in regulating inflammation-resolution, tissue regeneration, and resolving pain. These findings also suggest that chemical signals are shared in resolutive cellular-trafficking key in tissue regeneration across phyla.

The total organic synthesis of MaR1 was also achieved by Rodriguez and Spur using Sonogashira coupling (Rodriguez and Spur 2012a), who also reported resolvin D6 (Rodriguez and Spur 2012b) and synthesis of resolvin E2 (Rodriguez and Spur 2012c). Kobayashi et al. reported stereoselective total synthesis of protectin D1 (Ogawa and Kobayashi 2011), resolvin E2 (Ogawa et al. 2009), and resolvin E1 (Ogawa and Kobayashi 2009). The synthesis of the 18-HEPE precursor of E-series resolvins was also reported (Krishnamurthy et al. 2011). Importantly, the stereoselective actions of each SPM proved to be highly effective in regulating human PMN and monocytes in microfluidic chambers (Jones et al. 2012), establishing the translational potential of the novel SPM.

Maresin 1 is the first identified maresin. Recently, we investigated formation, stereochemistry, and precursor role of 13,14-epoxy-docosahexaenoic acid, an intermediate in MaR1 biosynthesis. The 14-lipoxygenation of DHA by human macrophage 12-lipoxygenase (hm12-LOX) gave 14-hydro(peroxy)-docosahexaenoic acid (14-HpDHA) as well as several dihydroxy-docosahexaenoic acids implicating an epoxide intermediate formation by this enzyme. Using a stereocontrolled synthesis, enantiomerically pure 13*S*,14*S*-epoxy-docosa-4*Z*,7*Z*,9*E*,11*E*,16*Z*,19*Z*-hexaenoic acid (13*S*,14*S*-epoxy-DHA) was prepared and its stereochemistry was confirmed by NMR spectroscopy. When this 13*S*,14*S*-epoxide was incubated with human macrophages, it was converted to MaR1. The synthetic 13*S*,14*S*-epoxide inhibited leukotriene B_4 (LTB_4) formation by human leukotriene A_4 hydrolase (LTA_4H) ~40 %, to a similar extent as LTA_4, but importantly was not converted to MaR1 by this enzyme. The 13*S*,14*S*-epoxide from DHA also reduced (~60 %) arachidonic acid conversion by hm12-LOX and promoted conversion of M1 macrophages to M2 phenotype, which produced more MaR1 from the epoxide than M1. These findings establish the biosynthesis of the 13*S*,14*S*-epoxide, its absolute stereochemistry, precursor role in MaR1 biosynthesis as well as its own intrinsic bioactivity (see Fig. 13.4). Given its actions and role in MaR1 biosynthesis, this epoxide was coined 13,14-epoxymaresin (13,14-eMaR) and possesses new mechanisms in resolution of

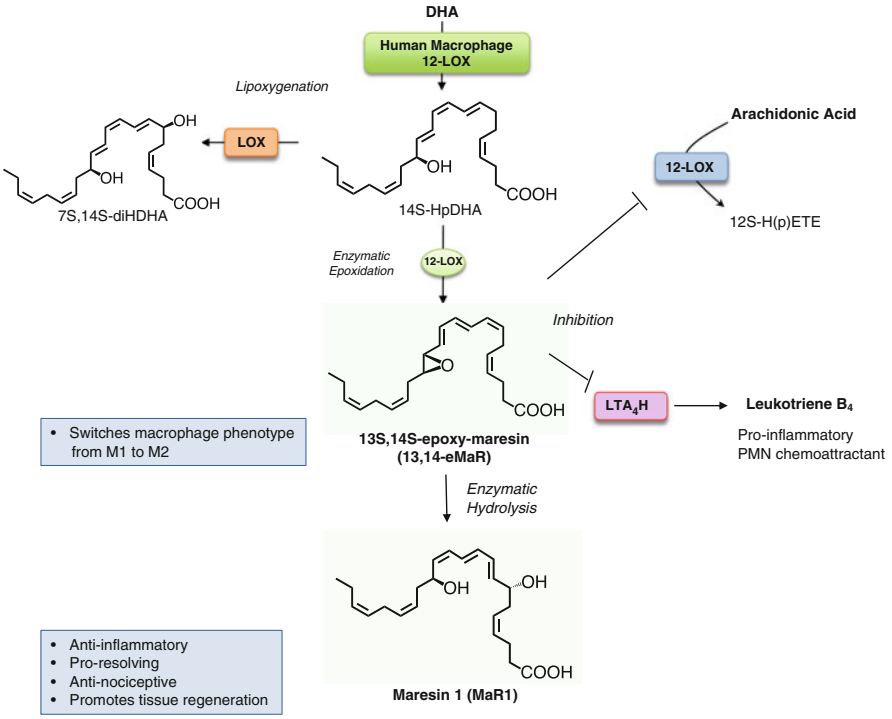

Fig. 13.4 DHA resolution metabolome—Maresin biosynthetic pathways. Biosynthetic scheme and actions of MaR1 and 13*S*,14*S*-eMaR. See Dalli et al. (2013b) and text for further details

inflammation by its ability to inhibit pro-inflammatory mediator production by LTA_4 hydrolase and to block arachidonate conversion by human 12-LOX (Dalli et al. 2013b). Hence, this enzyme regulates key events in resolution at multiple levels.

13.4 Operationalizing the Resolution Lipid Mediator Metabolome: Microparticles and Leukocyte Subpopulations

With many of the main SPM complete stereochemistry established it was next possible to carry out lipid mediator (LM) metabololipidomics profiling via LC-MS-MS-based analyses with distinct phagocyte populations, namely neutrophils (PMN), apoptotic PMN, and macrophages (Dalli and Serhan 2012). Efferocytosis increased SPM biosynthesis, including RvD1, RvD2, and RvE2 (Fig. 13.5), which were

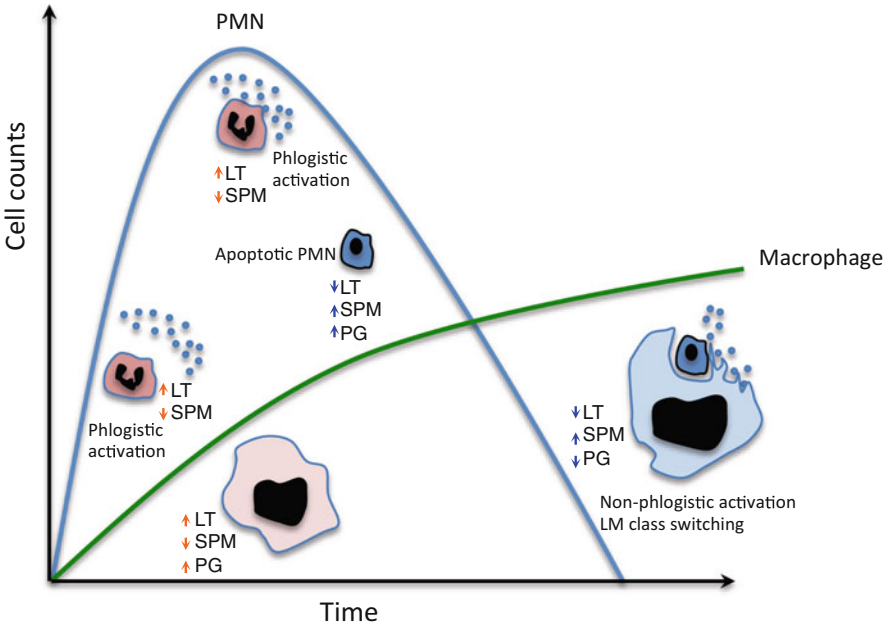

Fig. 13.5 Proposed regulation of macrophage lipid mediator phenotype during self-limited inflammation by neutrophils and neutrophil microparticles. Upon challenge neutrophils are recruited to the site of inflammation displaying a pro-inflammatory lipid mediator phenotype. As the inflammatory response proceeds monocytes/macrophages are also recruited that also display a pro-inflammatory lipid mediator phenotype with elevated leukotriene and prostanoid biosynthesis and lower pro-resolving mediator levels. As neutrophils undergo apoptosis there is a shift in the lipid mediator profile to a pro-resolving mediator profile displaying elevated prostaglandin and SPM levels as well as producing microparticles that carry precursors for SPM biosynthesis. These precursors, along with those carried by apoptotic cells are utilized by macrophages during efferocytosis to produce pro-resolving mediators leading to macrophage lipid mediator class switch from a pro-inflammatory to a pro-resolving phenotype with elevated SPM biosynthesis and reduced prostanoid and leukotriene levels. See Dalli and Serhan (2012) and text for further details

further elevated by PMN microparticles (Norling et al. 2011). Apoptotic PMN produced prostaglandin E_2, lipoxin B_4, and RvE2, whereas zymosan-stimulated PMN showed predominantly leukotriene B_4 and 20-hydroxy-leukotriene B_4, as well as lipoxin biosynthesis pathway marker 5,15-diHETE. Using deuterium-labeled precursors (d_8-arachidonic acid, d_5-eicosapentaenoic acid, and d_5-docosahexaenoic acid), we found that apoptotic PMN and microparticles each contribute to SPM biosynthesis during the process of efferocytosis. Also, the classic M2 macrophage phenotype (Lawrence and Natoli 2011) produces SPM including MaR1 with lower amounts of LTB_4 and PG than the M1 cells. Of interest, the uptake of apoptotic PMN by both macrophage subtypes led to modulation of their LM profiles and activation of transcellular SPM biosynthesis. LTB_4 was down regulated in M2 whereas SPM including LXA_4 are increased. These results establish LM signature profiles of human PMN, apoptotic PMN, and macrophage subpopulations. Moreover, they provide evidence for microparticle regulation of specific endogenous LM during defined stages of the acute inflammatory process and their dynamic changes in LM signatures via transcellular biosynthesis with apoptotic cells and microparticles.

13.5 Infection

We recently studied underlying mechanisms for how bacterial infections contribute to active resolution of inflammation. Exudate leukocyte trafficking and mediator-metabololipidomics of murine peritoneal *Escherichia coli* infections were carried out with temporal identification of both pro-inflammatory (prostaglandins and leukotrienes) and SPM. In self-resolving *E. coli* exudates (10^5 CFU), the dominant SPM identified were RvD5 and PD1, which at 12 h were significantly greater than levels in exudates from higher titer *E. coli* (10^7 CFU) challenged mice. Germ-free mice produced endogenous RvD1 and PD1 levels higher than in conventional mice. RvD1 and RvD5 (ng/mouse) each reduced bacterial titers in blood and exudates, *E. coli*-induced hypothermia, and increased survival. To translate these to humans, both human PMN and macrophages were tested and RvD1, RvD5, and PD1 each directly enhanced phagocytosis of *E. coli*, and RvD5 counter-regulated a panel of pro-inflammatory genes, including NF-κB and TNF-α. RvD5 activated the RvD1 receptor, GPR32, to enhance phagocytosis.

With self-limited *E. coli* infections, RvD1 and the antibiotic ciprofloxacin accelerated resolution, each shortening resolution intervals (R_i). Host-directed RvD1 actions enhanced ciprofloxacin's therapeutic actions. In 10^7 CFU *E. coli* infections, SPM (RvD1, RvD5, PD1) together with ciprofloxacin also heightened host antimicrobial responses. Also, in skin infections, SPM enhanced vancomycin clearance of *Staphylococcus aureus*. These results demonstrate that specific SPM are temporally and differentially regulated during infections and that they are anti-phlogistic, enhance containment, and lower antibiotic requirements for bacterial clearance. These endogenous resolution mechanisms are of interest in host defense because initiation of the host response is controlled by prostaglandins and leukotrienes (von Moltke et al. 2012), which when uncontrolled can lead to reduced survival from infection (Chiang et al. 2012) as observed in zebrafish infections (Tobin et al. 2012).

Also of interest, the SPM PD1 produced by the host was recently identified as a novel anti-viral that directly blocks viral replication and increases host survival to influenza viral infection (Morita et al. 2013).

13.6 SPM Receptors

13.6.1 Receptor Identification

ALX and GPR32 are 2 GPCRs for RvD1's on human phagocytes. ALX is the lipoxin A_4 receptor, and GPR32 was an orphan receptor. RvD1 displays specific binding and reduces actin polymerization and CD11b on PMN, as well as stimulate macrophage phagocytosis in an ALX and GPR32-dependent manner (Krishnamoorthy et al. 2010). In addition to RvD1, its aspirin-triggered epimer 17R-RvD1 and stable analog 17-R/S-methyl-RvD1 each dose-dependently activates ALX/FPR2 and GPR32 in GPCR-overexpressing β-arrestin systems and electric cell-substrate impedance sensing (Krishnamoorthy et al. 2012). Of interest, we demonstrated that RvD5 also activates human GPR32 in the GPR32-β-arrestin systems, and stimulates macrophage phagocytosis of *E. coli* in a GPR32-dependent manner (Chiang et al. 2012). In addition, RvD3 and AT-RvD3 each activates GPR32, contributing to their pro-resolving actions in stimulating macrophage uptake of microbial particles (Dalli et al. 2013a).

ChemR23, a specific receptor for RvE1, is closely related to lipoxin and leukotriene receptors in amino acid sequences. ChemR23 gave specific RvE1 binding, and RvE1-dependent signals to activate monocyte, and reduce dendritic cell migration and IL-12 production (Arita et al. 2005a). RvE1–ChemR23 interactions also stimulate macrophages phagocytosis via phosphorylation-signaling pathways including Ribosomal protein S6, a downstream target of the PI3K/Akt signaling pathway and the Raf/ERK pathway (Ohira et al. 2010). 18S-RvE1 also bind to ChemR23 with increased affinity and potency compared with the R-epimer, but was more rapidly inactivated than RvE1 (Oh et al. 2011). RvE2 is a partial agonist for ChemR23 (Oh et al. 2012). *BLT1*, a leukotriene B_4 receptor, also directly interacts with RvE1, that inhibits calcium mobilization, NF-κB activation in vitro, and PMN infiltration in vivo (Arita et al. 2007). 18S-RvE1 and RvE2 also bind to BLT1 (Oh et al. 2011, 2012). Therefore RvE1 gives cell-type specific actions, serving as an agonist for ChemR23 on mononuclear and dendritic cells as well as an antagonist for BLT1 signals on PMN. ChemR23-dependent actions of RvE1 are recently confirmed in mouse fibrosis (Qu et al. 2012).

13.6.2 Receptor Overexpression and Knockdown In Vivo

Transgenic (TG) mice. We constructed mice overexpressing human ALX. hALX transgene was placed under the control of CD11b promoter, directing receptor expression in myeloid cells. In non-TG littermates, RvD1 as low as 10 ng given together

with zymosan, reduced leukocyte numbers by ~38 % at 24 h. This action was further enhanced in ALX-TG mice giving 53 % reduction of leukocytes. Also with RvD1 treatment, PMN numbers in TG mice were 50 % lower than non-TG controls (Krishnamoorthy et al. 2012). Lipid mediator-metabololipidomics carried out with 24-h exudates revealed that RvD1 in vivo gave a significant reduction in a number of pro-inflammatory mediators including prostaglandins and LTB_4 in wild-type mice. We also prepared transgenic mice overexpressing human ChemR23, the RvE1 receptor, on myeloid cells. In these TG mice, RvE1 is tenfold more potent in limiting PMN infiltration in zymosan-initiated peritonitis, compared with non-TG littermates. In addition, ligature-induced alveolar bone loss was diminished in ChemR23tg mice. Local RvE1 treatment of uniform craniotomy in the parietal bone significantly accelerated regeneration of the bone defect. These results indicate that RvE1 modulates osteoclast differentiation and bone remodeling by direct actions on bone, in addition to its anti-inflammatory and pro-resolving actions (Krishnamoorthy et al. 2012).

Receptor knockout mice. In mice deficient of fpr2/ALX (mouse orthologue of human ALX), the anti-inflammatory actions of RvD1 were abolished. Administration of RvD1 (1 ng/mouse) significantly reduces PMN infiltration in wild-type mice, but not in fpr2 null mice. Also in peritoneal exudates, RvD1 activates lipoxin biosynthesis stimulating the production of the anti-inflammatory mediator LXB_4 and stimulated the biosynthesis of the cyclooxygenase-derived PGE_2 while downregulating production of the pro-inflammatory LTB_4. This regulation of lipid mediator by RvD1 is lost in the fpr2 null mice (Norling et al. 2012). These results indicate that RvD1 dampens acute inflammation in part via ALX receptor. In BLT1 knockout mice, in vivo anti-inflammatory actions of RvE1 were sharply reduced when given at low doses (100 ng i.v.) in peritonitis. In contrast, RvE1 at higher doses (1.0 μg i.v.) significantly reduced PMN infiltration in a BLT1-independent manner. These results indicate that RvE1 binds to BLT1 as a partial agonist, serving as a local damper of BLT1 signals on leukocytes along with other receptors (e.g., ChemR23 receptor-mediated counterregulatory actions) to mediate the resolution of inflammation (Arita et al. 2007).

13.7 Organs of Actions

13.7.1 Ocular inflammation – Clinical Development

Mouse: In human and rat conjunctiva goblet cells, RvD1 and RvE1 reduce LTD_4- and histamine-stimulated conjunctival goblet cell secretion (Dartt et al. 2011; Li et al. 2013). In a murine model of dry eye, RvE1 delivered as its methyl ester improves the outcome measures of corneal staining and goblet cell density, indicating the potential utility of resolvins in the treatment of dry eye (de Paiva et al. 2012). In HSV-induced ocular inflammation, RvE1 significantly reduces cornea lesions and angiogenesis as well as T cells and PMN. These results indicate that RvE1

represents a novel approach to control virus-induced diseases (Rajasagi et al. 2011). A recent study demonstrated a phenotype of delayed wound healing in cornea of female mice. Also in human corneal epithelial cells, estradiol reduced 15-LOX type-I and LXA_4. LXA_4 addition rescues the estradiol-abrogated wound healing, demonstrating gender-specific differences in the corneal repair mediated by the 15-LOX-LXA_4 circuit (Wang et al. 2012). In uveitis in rats, bolus intravenous injection of RvD1 (10–1,000 ng/kg) significantly and dose-dependently reduced LPS-induced ocular derangement and PMN, T-lymphocytes as well as cytokines within the eye (Settimio et al. 2012).

Human. In a combined Phase 1 and phase 2 clinical trial in patients with dry eye syndrome, a RvE1 analog significant improved signs and symptoms. This is the first demonstration of clinical efficacy for the novel class of resolvin therapeutics. The phase III clinical trial is now in progress (Safety and Efficacy Study of RX-10045 on the Signs and Symptoms of Dry Eye, identifier NCT00799552; URL: http://www.clinicaltrials.gov).

Salivary—Sjögren's syndrome. Exposure of salivary epithelium to TNFα and/or IFNγ alters tight junction integrity, leading to secretory dysfunction. RvD1 (100 ng/ml) rescues TNFα-induced tight junction and cytoskeletal disruption, and enhances cell migration and polarity in an ALX-dependent manner. These findings suggest that RvD1 promotes tissue repair in salivary epithelium and restores salivary gland dysfunction associated with Sjögren's syndrome (Odusanwo et al. 2012).

Oral inflammation. In localized aggressive periodontitis (LAP) patients, macrophages exhibit reduced phagocytosis. RvE1 rescues impaired phagocytic activity of LAP macrophages (Fredman et al. 2011). Humanized nanoparticles containing 17R-RvD1 or LXA_4 analog protect against inflammation in the temporomandibular joint, a model of temporomandibular joint disease (Norling et al. 2011).

Vascular inflammation. In a rabbit model of arterial angioplasty, endogenous biosynthesis of pro-resolving lipid mediators, including RvD5 and LXB_4 was found in artery wall. RvD2 attenuated cell proliferation, leukocyte recruitment, and neointimal hyperplasia (Miyahara et al. 2013).

Airway inflammation. In allergic airway inflammation, RvE1 promotes resolution in part via suppressing IL-23 and IL-6 as well as increasing IFN-γ (Haworth et al. 2008). Also, RvE1 regulates NK cell migration and cytotoxicity (Haworth et al. 2011). The DHA-derived AT-RvD1 and RvD1 each markedly shortens the resolution intervals for lung eosinophilia and reduces select inflammatory peptides and lipid mediators (Rogerio et al. 2012). In acute lung injury, AT-RvD1 improves epithelial and endothelial barrier integrity, decreases airway resistance, and increases epinephrine levels in bronchoalveolar lavage fluid (BALF) (Eickmeier et al. 2013). Of interest, Fat-1 transgenic mice that have increased endogenous lung n-3 PUFA (Hudert et al. 2006) also show higher PD1 and RvE1 levels after bronchoprovocative challenge. These animals suggest a protective role for endogenous SPM in allergic airway responses, decreasing airway inflammation with reduced leukocyte

accumulation in BALF and lung parenchyma (Bilal et al. 2011). It is noteworthy that human eosinophils generate PD1 as one of the main pro-resolving molecules, and PD1 production by eosinophils is impaired in patients with severe asthma. PD1, in nanomolar concentrations, reduces eosinophil chemotaxis and adhesion molecules (Miyata et al. 2013). Recently, RvD1 was shown to protect cigarette smoke-induced lung inflammation, significantly reduced PMN infiltration and promoted differentiation of M2 macrophages and efferocytosis in vivo. RvD1 also accelerated resolution of lung inflammation, demonstrating strong potential to resolve lung injuries caused by smoke and pulmonary toxicants (Hsiao et al. 2013).

Pain and neuroinflammation. Resolvins are potent regulators of inflammatory pain (Xu et al. 2010). Also, intrathecal injections of RvD1 (40 ng) in rats strongly reduces postoperative pain (Huang et al. 2011). RvE1, via intrathecal injection prevents nerve injury-induced mechanical allodynia in spinal cord dorsal horn (Xu et al. 2013). Along these lines, RvD1 (100 ng/kg) significantly decreases TNBS-induced mechanical allodynia and blocked cytokine production in spinal dorsal horn (Quan-Xin et al. 2012). RvD2 (0.01–1 ng) prevents formalin-induced spontaneous pain. As part of the molecular mechanisms, RvD2, RvE1, and RvD1 differentially regulated transient receptor potential (TRP) channels (Park et al. 2011). AT-RvD1 significantly reverses the thermal hypersensitivity and knockdown of epidermal TRPV3 blunts these anti-nociceptive actions (Bang et al. 2012). In arthritis, AT-RvD1 exhibits marked anti-hyperalgesia, decreases production of TNF-α and IL-1β in rat hind paw (Lima-Garcia et al. 2011). RvD1 also attenuates neuroinflammation, stimulating phagocytosis of amyloid-β (Aβ) by Alzheimer's disease macrophages and inhibits fibrillar Aβ-induced apoptosis in a GPR32-dependent manner (Mizwicki et al. 2013).

Dermatologic SPM actions. *Mouse*. PMN infiltration to mouse skin was used for the structure elucidation of resolvins (Serhan et al. 2000) that proved critical for development. In mouse burn models, RvD2 at 25 pg/g/animal given systemically post burn effectively prevents thrombosis of the deep dermal vasculature, dermal necrosis, and PMN-mediated damage (Bohr et al. 2013). Additionally, RvD2 restored PMN directionality and increased survival post burn after a second septic insult. Thus, RvD2 could have therapeutic potential delaying lethal complications after burn injuries (Kurihara et al. 2013). In DNFB-stimulated dermatitis skin lesions, RvE1 reduces skin lesions by lowering IL-4 and IFN-γ in activated CD4(+) T cells as well as serum IgE (Kim et al. 2012).

Human. Topical treatment of a LXA_4 stable analog 15(R/S)-methyl-LXA_4 significantly reduces the severity of eczema in a two-centers, double-blind, placebo-controlled, randomized, parallel-groups comparative study with 60 infants (Wu et al. 2013).

Gastrointestinal tract. In murine colitis, systemic RvE1, AT-RvD1, RvD2, or 17R-HDHA in nanogram ranges improve disease severity, prevent body weight

loss, colonic damage, and PMN infiltration as well as lower select colonic cytokines. The results suggest that some of the SPM have the potential in treating inflammatory bowel diseases (Arita et al. 2005b; Bento et al. 2011).

Fibrosis. In a unilateral ureteric obstruction (UUO)-driven murine fibrosis, administration of RvE1 at only 300 ng/day/mouse reportedly reduces accumulation of myofibroblasts, deposition of collagen IV, and myofibroblast proliferation. RvE1 (~1–30 nM) directly inhibits PDGF-BB-induced proliferation in mouse fibroblasts in a ChemR23 receptor-dependent fashion (Qu et al. 2012).

Metabolic syndromes. RvD1 stimulates nonphlogistic phagocytosis in adipose macrophages and reduces macrophage reactive oxygen species production (Titos et al. 2011). Also, in leptin receptor-deficient (db/db) mice, RvD1 (2 μg/kg) improves glucose tolerance, decreases fasting blood glucose, increases adiponectin production and markers of alternatively activated M2 macrophages (Hellmann et al. 2011). Alone these lines, LXA_4 [1 nM] also attenuates adipose inflammation and improves insulin sensitivity in a model of age-associated adipose inflammation (Borgeson et al. 2012). In diabetic wounds, local application of RvD1 accelerates wound closure and reduces accumulation of apoptotic cells in the wounds (Tang et al. 2013). It is noteworthy that in streptozotocin (STZ)-induced diabetes, fat-1 transgenic mice do not develop hyperglycemia and β-cell destruction compared with wild-type mice. RvE1 levels are highly increased in these fat-1 mice, emphasizing endogenous roles for RvE1 in diabetes (Bellenger et al. 2011). Thus, select SPM or their mimetics may be novel therapeutics, reducing adipose inflammation and insulin resistance, which are the key components of type 2 diabetes.

13.8 Micro RNAs of Resolution: SPM-GPCR-microRNA Circuits

RvD1 accelerates resolution and controls specific miRNA expression including miR-146b, 208a, and 219 (Recchiuti et al. 2011). This panel of miRs was temporally regulated during self-limited inflammation and controlled by RvD1 in vivo as well as in a RvD1-GPCR-dependent manner in human macrophages (Recchiuti et al. 2011). Macrophages overexpressing miR-219 significantly down-regulate 5-LOX and reduce LTB_4. Hence, 5-LOX is a target gene of miR-219 (Recchiuti et al. 2011). In addition, RvD1 at low dose (10 ng) significantly increases miR-219 in ALX TG mice, whereas this dose of RvD1 was not effective in non-TG controls (Krishnamoorthy et al. 2012). Of note, delayed resolution initiated by high-dose zymosan challenges decreases miR-219-5p expression along with higher LTB_4 and lower SPM (Fredman et al. 2012). Thus, both ALX and miR-219 are components of RvD1-initiated resolution circuits (Fig. 13.6).

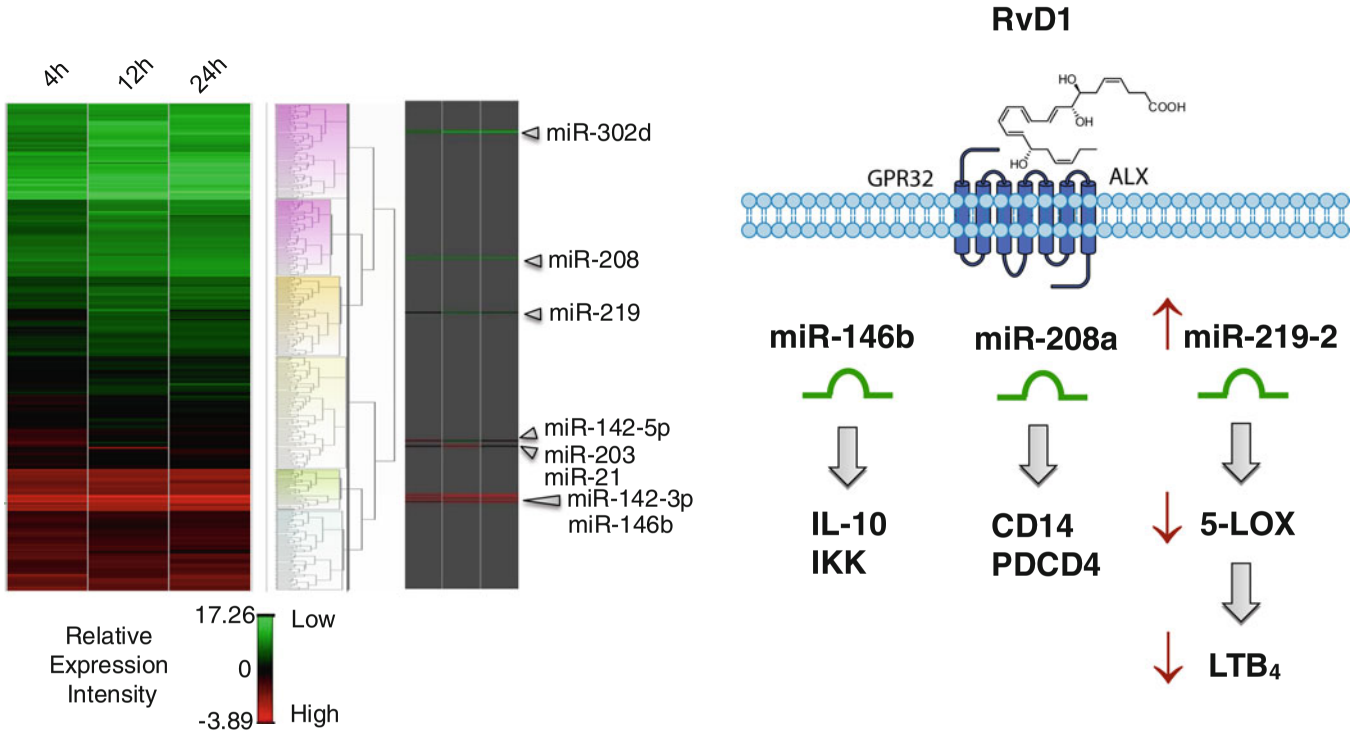

Fig. 13.6 RvD1-GPCR-microRNA axis accelerating resolution. A panel of microRNAs is temporally regulated during self-limited inflammation, and controlled by RvD1 in vivo (Recchiuti et al. 2011). In addition, these miRs are regulated in a RvD1-GPCR-dependent manner in human macrophages. Among them, miR-219 significantly down-regulates 5-LOX expression and reduces 5-LOX product LTB_4, determined by LC-MS-based metabolomics. It is likely that these miRNAs and some of their target genes contribute to RvD1's pro-resolving actions in vivo

13.9 Conclusions

Professor Rod Flower of the William Harvey Research Institute, University of London recited the following quotation from the Roman poet Juvenal to introduce these new findings in London on 20 July 2011: ***Quis custodiet ipsos custodes***? Who will guard the guards themselves?

Pus bonum et laudabile—from ancient Latin "good and laudable pus" (Majno 1982; Serhan 2004) and namely the PMN or so-called white blood cells' innate phagocytes; the guards themselves. From research in this laboratory, it is now clear that within pus endogenous molecules are present that are beneficial that we now call resolvins, protectins, and maresins (the SPM), which terminate inflammation and stimulate resolution. As reviewed in this chapter, recently the complete stereochemistries of several additional SPM were obtained and several teams have published on their total organic synthesis, confirming the novel structures and actions. With availability of synthetic resolvins, in recent years a body of literature emerged that expands their potent pro-resolving and anti-inflammatory actions on phagocytes (PMN and MΦ) into many disease models. A few representative SPM have now entered into human trials and are reported to be effective in several conditions: skin inflammation and ocular disease. They offer a new class of therapeutics that are *agonists* rather than enzyme inhibitors or receptor antagonists to treat excessive inflammation. These new findings together provide further support for the novel concept that return of acute inflammatory responses to homeostasis involves active biosynthesis of pro-resolving local autacoids. Moreover, these novel receptor agonists activate resolution programs that return tissues to homeostasis and regenerate function. Given their ubiquitous production in vivo, it is likely that the SPM will have pivotal actions in many other important organ systems and physiologic and pathophysiologic processes.

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Chapter 14
Macrophages in Sepsis Progression

Eduardo López-Collazo, Jean-Marc Cavaillon, and Subhra K. Biswas

14.1 Sepsis: History and Epidemiology

Sepsis is a complex syndrome characterized by a dysregulated inflammatory response to systemic infection. It is a multifarious pathology that is one of chief causes of death in intensive care units (ICUs) worldwide (Hotchkiss et al. 2009). In the past, sepsis mainly occurred due to infection developing from wounds received on the battlefield. Another frequent occurrence of sepsis was puerperal fever in women, following delivery. By the end of the eighteenth century, Alexander Gordon of Aberdeen claimed that puerperal fever was an infectious and contagious disease. In 1847, Ignaz Semmelweis, a Hungarian doctor working in Vienna successfully demonstrated this was indeed the case, and succeeded, by antiseptic methods, in reducing the mortality due to puerperal sepsis from 16 % to less than 1 % in his clinic. But he was not trusted till the work of Louis Pasteur who also advocated antiseptic methods and hygiene in 1879. In the meantime, Victor Feltz and Léon Coze, two Alsatian doctors had discovered that there were bacteria within the blood of a woman who died of puerperal fever. Patients with puerperal fever were the first humans in whom antibiotics (prontosil) were tested and shown to be successful in 1936.

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Nowadays, sepsis mainly affects the very early and late stage of life. One person dies of sepsis in the world every 3–4 s. In industrialized countries, sepsis represents as many deaths as myocardial infarction. There are 377 cases per 100,000 inhabitants (The World Sepsis Day Fact Sheet, September 13, 2012). The mortality of patients with sepsis is 20–30 %, while that of the most severe form (i.e., septic shock) can reach 50 % (Annane et al. 2005). The future projection suggests a doubling of the number of cases over the next 50 years, especially due to aging of the population.

14.2 Sepsis: Definition and Complexity

Sepsis is an infectious systemic inflammatory response syndrome (SIRS). SIRS is defined by at least two parameters from the following list: Fever (>38 °C) or hypothermia (<36 °C), tachypnea (>24 breaths/min), tachycardia (>90 beats/min), leukocytosis (>12,000/mm) or leucopenia. Severe sepsis is a sepsis with organ failure, and septic shock is a severe sepsis with a refractory hypotension. Sepsis can occur in patients in ICU with SIRS (e.g., severe surgery, trauma, pancreatitis, resuscitated cardiac arrest, burns). The earlier the antibiotic therapy is initiated, the greater will be the chance to survive sepsis (Kumar et al. 2006). However, as stated by William Osler at the beginning of the twentieth century: "Except on few occasions, the patient appears to die from the body's response to infection rather than from it." Indeed, the host response in sepsis is characterized by an early "Cytokine storm" that reflects the exacerbated production of inflammatory mediators. However, a key feature of sepsis is its complexity. Accordingly, both inflammatory and anti-inflammatory features are associated with the disease. Roger Bone coined a new concept, the compensatory anti-inflammatory response syndrome (CARS) (Adib-Conquy and Cavaillon 2009). In contrast to SIRS which is characterized by leukocyte activation, cytokine storm, systemic inflammation, and tissue injury, CARS involves leukocyte deactivation, immunosuppression, endothelial/epithelial dysfunction, and inability to response to further endotoxin challenge and/or infections (Fig. 14.1). In fact, sepsis-induced immunosuppression has been linked to increased risks of nosocomial infection and mortality in most sepsis patients (Pachot et al. 2006; Monneret et al. 2008). In line with the above, in a cohort of 464 septic patients, van Dissel et al. (1998) reported that a high ratio of IL-10 to TNFα correlated with mortality. Similarly, transcriptomic analysis of sepsis patients showed downregulation of HLA-DR (indicative of immunosuppression) to correlate with mortality (Monneret et al. 2004; Pachot et al. 2006). Other authors documented impaired pro-inflammatory response of sepsis mononuclear cells to ex vivo activation, while survival was associated with the recovery of the pro-inflammatory, but not anti-inflammatory response (Munoz et al. 1991a; Ertel et al. 1995; Rigato and Salomao 2003; Sinistro et al. 2008).

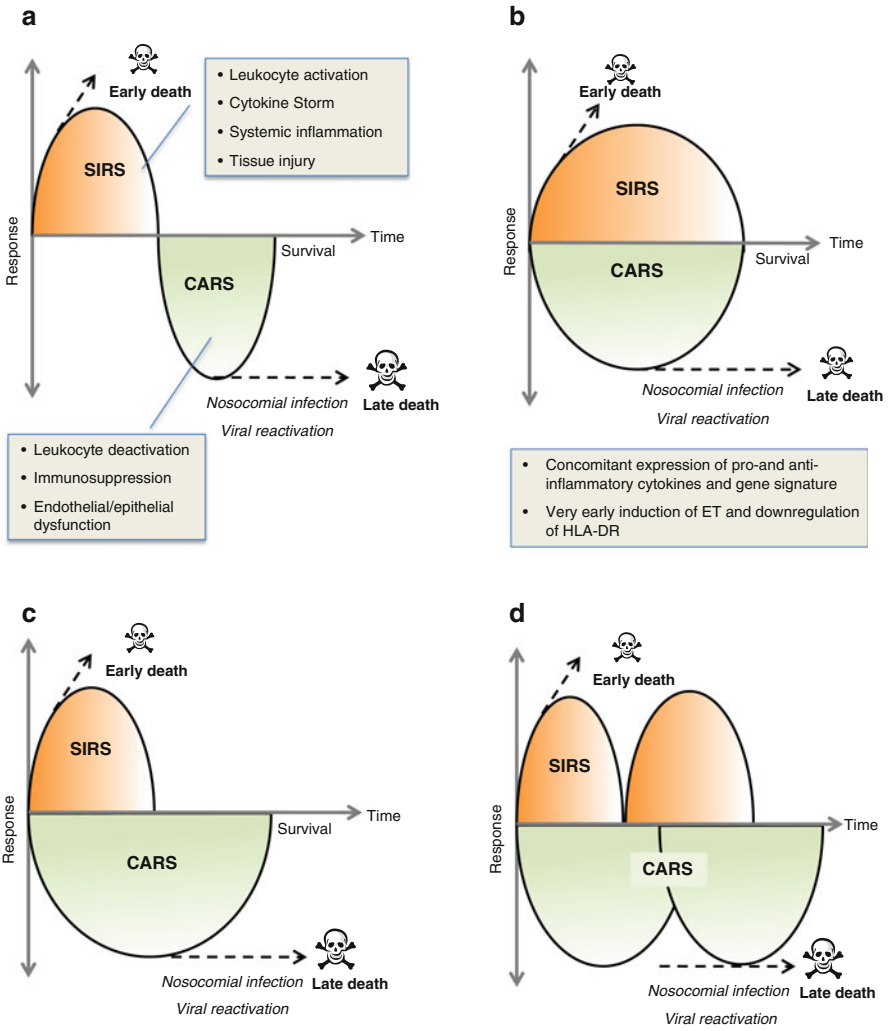

Fig. 14.1 Immune response in sepsis. Immune response in sepsis consists of both pro-inflammatory and anti-inflammatory (or immunosuppressive) features. SIRS constitutes the pro-inflammatory response while CARS constitutes the anti-inflammatory or immunosuppressive response. (**a**) According to the existing dogma, SIRS defines the initial phase in sepsis, which is followed by CARS. Features of SIRS and CARS are summarized in the text boxes within the figure; (**b**) After insult, SIRS and CARS are initiated concomitantly in sepsis patients. Some evidences for their concomitant occurrence are presented in the text box below the figure; (**c**) After the insult, while SIRS may be resolved in some patients, their immune status might be altered over a long period of time; (**d**) After a first insult, a second one (such as a nosocomial infection) can occur, leading to a second SIRS and a further occurrence of the CARS status. Figure adapted from Hotchkiss et al. (2013) and Cavaillon et al. (2001)

CARS should be considered as an "adapted" compartmentalized response with the aim to silence some acute pro-inflammatory genes, and to maintain the possible expression of certain genes involved in the anti-infectious process (Adib-Conquy and Cavaillon 2009). Although it has been claimed that SIRS precedes CARS (Fig. 14.1a), this may not be the case (Cavaillon et al. 2001). First, the plasma levels of IL-10, the main anti-inflammatory cytokine fully correlates with those of inflammatory cytokines (e.g., TNF, IL-6, IL-8) (Gomez-Jimenez et al. 1995; Lehmann et al. 1995; van Deuren et al. 1995; Kasai et al. 1997; Cavaillon et al. 2003). Of course, this could not be the case if CARS would occur after SIRS. In fact, pro- and anti-inflammatory cytokines are regulated simultaneously from the first moments of septic shock (Tamayo et al. 2011). Second, reduced ex vivo cytokine production can be immediately observed after admission of resuscitated cardiac arrest patients in ICU (Adrie et al. 2002) or during the operation in surgery patients (Versteeg et al. 2009). Thirdly, the reduced expression of HLA-DR on monocytes, a hallmark of CARS (see below), also occurs during the time of the operation in surgery patients (Kim et al. 2010), and was already reduced on monocytes taken very soon after severe trauma at the accident scene (Kox et al. 2013). Fourthly, gene expression profiling of human circulating cells in sepsis has failed to demonstrate the existence of a distinct pro-inflammatory and anti-inflammatory phase at the transcriptional level (Tang et al. 2010). In support, a recent transcriptome study in severely ill patients also indicated the immune response to consist of both pro-inflammatory and anti-inflammatory aspects, functioning in parallel (Xiao et al. 2011). Taken together, all these evidence emphasize the concomitant occurrence of pro-inflammatory and anti-inflammatory events in sepsis (Cavaillon et al. 2001; Hotchkiss et al. 2013) (Fig. 14.1b–d). Adding further to this complexity, recent studies indicate species-specific differences in the inflammatory response and its innate immune mechanisms between humans and mice, making it difficult to extrapolate findings from preclinical models to humans (Schroder et al. 2012; Seok et al. 2013). Finally, endothelial and epithelial dysfunctions, vascular leakage, organ failure, and divergent immune response in the different tissues (as compared to blood) present additional complications that remain to be characterized in sepsis.

14.3 Monocytes and Macrophages as Mediators of Immuno-Inflammatory Response During Sepsis

Monocytes and macrophages are key components of the innate immune system and serve as the first line of defense against infections. These cells show phenomenal functional diversity [(Taylor and Gordon 2003; Biswas and Mantovani 2010); detailed in Chap. 2], triggering inflammation, phagocytosis, and microbicidal activity to fight an infection, while resolving inflammation and promoting tissue repair to re-instate tissue homeostasis upon clearance of the infection. In the same vein, monocytes and macrophages also contribute to a plethora of functions that orchestrate sepsis progression (discussed below). It is important to note that during sepsis,

profound apoptosis of lymphocyte subsets (e.g., B-cells, T-cells) and dendritic cells, but not monocytes or macrophages have been reported (Hotchkiss et al. 2001, 2002). This leaves monocytes and macrophages as a key immune cell type that regulates the immune response during sepsis and a possible target for therapeutic modulation.

During an infection, monocyte/macrophages come in contact with the invading pathogen and its various microbial components (e.g., components of the bacterial wall, nucleic acids, etc.), collectively referred to as *p*athogen-*a*ssociated *m*olecular *p*atterns (PAMPs). These PAMPs are recognized by *p*atterns *r*ecognition *r*eceptors (PRRs) such as *T*oll-*l*ike *r*eceptors (TLRs), present on the surface of monocytes and macrophages. This triggers several intracellular signaling pathways that culminate in the activation of transcription factors such as *N*uclear *F*actor-Kappa B (NF-κB) which regulates the expression of a plethora of inflammatory cytokines (e.g., TNFα, IL-8, IL-6, IL-1, etc.). Such a pro-inflammatory phenotype is reminiscent of the classical (or M1) activation state for monocyte/macrophages (Fig. 14.2).

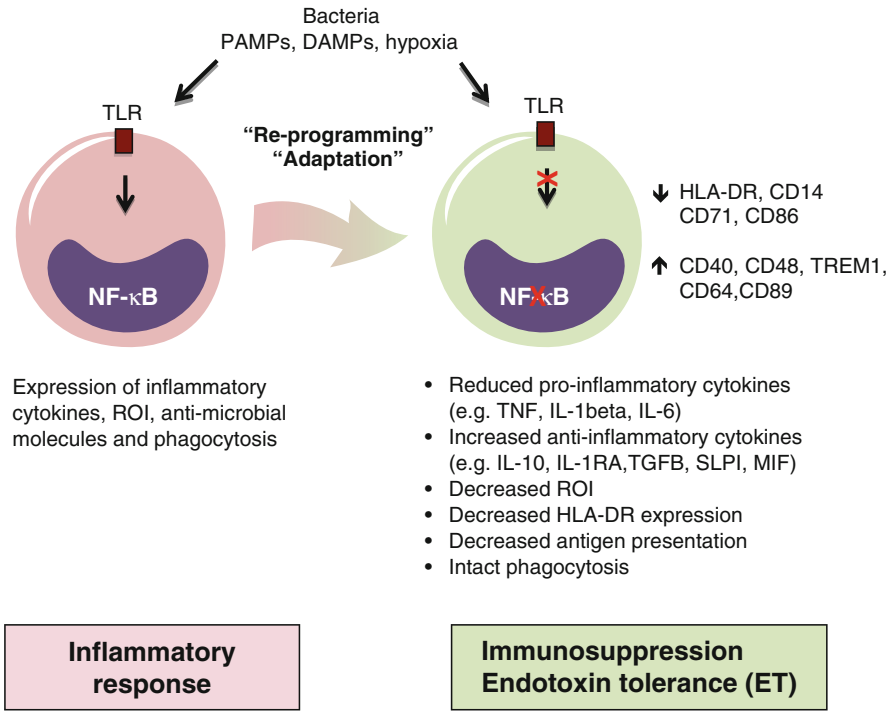

Fig. 14.2 Functional re-programming of monocyte–macrophage in sepsis. Monocyte–macrophages undergo a functional re-programming during the course of sepsis. They initially exhibit a pro-inflammatory response to microbes, PAMPs and DAMPs resulting in the release of pro-inflammatory cytokines, generation of reactive oxygen intermediates (ROI) and phagocytosis. However, these cells subsequently undergo re-programming wherein they show endotoxin tolerance, i.e., inability to express pro-inflammatory cytokines, generate ROI, and instead, upregulate expression of anti-inflammatory cytokines, upon endotoxin re-challenge. However, they maintain intact phagocytic capability

During sepsis, activation of monocyte/macrophages are not only triggered by their interaction with the PAMPS, but also by components released by injured tissues and necrotic cells, collectively referred to as the *d*amage *a*ssociated *m*olecular *p*atterns (DAMPs). Besides the PAMPs and DAMPs, these cells also receive other signals released during the inflammatory process, such as anaphylatoxin C5a, lipid mediators, cytokines, neuro-mediators, coagulation factors, hemoglobin, and hypoxia, that act in synergy to enhance the release of cytokines (Adib-Conquy and Cavaillon 2007). The role of macrophages and their associated cytokines remains ambivalent as illustrated by studies with mice deficient in Macrophage-Colony Stimulating Factor (M-CSF). These M-CSF-deficient mice were more sensitive to *Escherichia coli*-induced peritonitis, but more resistant to an lipopolysaccharide (LPS) injection (Wiktor-Jedrzejczak et al. 1996).

In animal models, monocytes have been rarely investigated as a source of cytokines during sepsis, SIRS or endotoxemia. After a systemic delivery of LPS, inflammatory cytokines are produced in all tissues: the brain (Qin et al. 2007), the heart (Kadokami et al. 2001), the kidney (Zager et al. 2007), the lungs (Hacham et al. 1996), the liver (Chensue et al. 1991), the bone marrow (Schmauder et al. 1994), the bowel (Ulich et al. 1992), and the spleen (Villa et al. 1995). Interestingly, the relative contribution of each organ is different for different cytokines (Hviid et al. 2012). Bronchoalveolar cells, splenocytes, and peritoneal cells harvested 1 h after an LPS injection release enhanced levels of TNF (Fitting et al. 2004). The cecal ligation and puncture (CLP) is a widely used experimental model of murine sepsis. Spleen and liver are the main sources of inflammatory cytokines during CLP (Wang et al. 1997; Sun et al. 2001). In particular, mononuclear phagocytes like Kupffer cells (Koo et al. 1999), alveolar macrophages (Meng et al. 1993), and peritoneal macrophages (McMasters and Cheadle 1993) are activated and produce inflammatory cytokines during CLP. These cells are also source of inflammatory cytokines in sterile SIRS such as hemorrhage or trauma (Zhu et al. 1994; Neunaber et al. 2013). Of note, a possible role may also exist for infiltrating macrophages within fat tissues (Langouche et al. 2010).

In contrast to murine models, in sepsis patients, monocytes have been the main studied mononuclear phagocytes. Taking advantage of the accumulation of IL-1 within the activated monocytes, the production of this cytokines in circulating monocytes was observed in some circumstances such as during extra-corporal circulation (Haeffner-Cavaillon et al. 1989). A similar approach allowed the detection of IL-1 associated with monocytes in less than 25 % of sepsis patients at admission (Munoz et al. 1991b). Surprisingly more than 80 % of patients had monocyte-associated TNF. This does not mean that monocytes are the principal source of TNF during sepsis since in many studies authors failed to detect TNF mRNA in these cells. Most probably, monocytes are bathing in a milieu that does contain these cytokines and they can capture them via their high affinity receptors. This hypothesis is illustrated by the presence of intracellular IL-10 in monocytes after trauma in all patients, whereas the IL-10 mRNA was detected in only 50 % of the patients (Shimonkevitz et al. 1999). Similarly, high levels of leukocyte associated IL-8 have been observed in circulating cells of sepsis patients (Marie et al. 1997), and this

cytokine is also produced by circulating leukocytes as mRNA have been detected in these cells (Friedland et al. 1992). In fact, most probably, once activated, monocytes would not remain within the bloodstream and would migrate to the tissues. This explains why monocytes are not considered as the major source of plasma cytokines in sepsis patients (Gille-Johnson et al. 2012), but other sources have been poorly characterized in humans. In patients with acute respiratory distress syndrome, most alveolar macrophages express TNF mRNA (Tran Van Nhieu et al. 1993) and an increased nuclear expression of NF-κB (Schwartz et al. 1996; Moine et al. 2000).

14.4 Functional Phenotype of Monocyte–Macrophage in Sepsis

During the incidence of sepsis, monocyte/macrophages respond to bacteria, endotoxins, and other associated danger signals to express various pro-inflammatory mediators that contribute to the ensuing "Cytokine storm." Concomitant with this overt inflammation, these cells also undergo an "adaptation" or "re-programming" wherein they start developing immunosuppressive features (Fig. 14.2). One of the best-known examples for this is their refractoriness to endotoxin challenge, a phenomenon described as "Endotoxin Tolerance" (ET) (Cavaillon et al. 2003; Biswas and Lopez-Collazo 2009). Blood monocytes from sepsis patients exhibit characteristics of endotoxin tolerance (Escoll et al. 2003; Biswas and Lopez-Collazo 2009). These monocytes, in contrast to monocytes from healthy volunteers, fail to produce pro-inflammatory cytokines such as TNFα, upon ex vivo endotoxin challenge (Munoz et al. 1991a, b; Monneret et al. 2008; Draisma et al. 2009). Another important immunosuppressive feature is the downregulation of MHC class II (HLA-DR), CD86, and CIITA (Manjuck et al. 2000; Pachot et al. 2006). However, enhanced expression of anti-inflammatory factors such as transforming growth factor-β (TGF-β), IL-1 receptor antagosnist (IL-1RA), secretory leukocyte protease inhibitor (SLPI), Migration inhibitory factor (MIF), and glucocorticoids has been reported for these cells and believed to contribute to the refractory state of the septic patients (Nathan 2002; Cavaillon and Adib-Conquy 2006; Monneret et al. 2008; Adib-Conquy and Cavaillon 2009). In addition, the phagocytosis activity of sepsis macrophages remains unchanged (Danikas et al. 2008) and so does the expression of numerous cell surface markers (e.g. CD40, CD48, CD64, CD80, CD89, Tissue factor, p55TNFR) (Adib-Conquy and Cavaillon 2009).

Collectively, the downregulation of inflammatory cytokines, upregulation of anti-inflammatory mediators, and intact or heightened phagocytic capacity suggest an M2-like polarization state for monocyte/macrophages in sepsis, as has described in endotoxin tolerance (Porta et al. 2009; Pena et al. 2011). However, assigning a definitive polarization phenotype to monocytes in sepsis patients is difficult in view of the overlapping pro-inflammatory and immunosuppressive features shared by these cells. But, definitely, the data presented above supports the idea of a functional and phenotypic plasticity of these cells in course of sepsis (Fig. 14.2).

Genome-wide sequencing or transcriptome analysis coupled with the assessment of monocyte/macrophage function in sepsis patients should shed light on the phenotype of these cells. However, such studies need to take into consideration the stage of sepsis as well as the type of insult and/or infection involved. In this context, it may be noted that downregulation of inflammatory cytokines noted in sepsis monocytes has been found to be dependent on the type of insult they are exposed to. For example, ex vivo TNF production in response to heat-killed *Staphylococci* is unaltered in sepsis and non-infectious SIRS (Adrie et al. 2002; Adib-Conquy et al. 2003, 2006). Similarly, another ex vivo observation on monocyte cell line demonstrated that LPS and *Staphylococcus aureus* induced heterologous priming instead of a cross-tolerance (Peck et al. 2004). Furthermore, the altered ex vivo TNF production is not observed for all mononuclear phagocytes. When exposed to LPS, alveolar macrophages from ARDS patients display enhanced IL-1 production (Jacobs et al. 1989). In parallel, peritoneal macrophages from patients with peritoneal infection are primed in vivo to release an increased amount of IL-1 beta in vitro after subsequent exogenous stimulation with LPS (Fieren et al. 1990). These observations indicate the heterogeneity of monocyte–macrophage response in sepsis depending on the compartment they belong to. In fact, as opposed to the numerous studies on circulating leukocytes and monocytes in sepsis patients, very little is known about the phenotype of tissue macrophages in different organs during sepsis. Inflammation in vital organs and their consequent failure is an important event in sepsis (Duffield 2010). Thus, studying the role of tissue macrophages in this process will provide important insight into the cellular mechanism of sepsis.

14.5 Mononuclear Phagocytes in Different Compartments Show Divergent Response to Infection and Sepsis

There are many differences between monocytes and macrophages depending upon the tissue they are derived from. For example, lung, peritoneum, spleen, microglia macrophages, and Kupffer cells show differences in gene expression profile and cell surface markers (Gorgani et al. 2008; Gautier et al. 2012). Intestinal macrophages despite possessing normal phagocytic and bactericidal activity are unable to produce cytokines upon activation (Smythies et al. 2005). The mechanisms involved in TNF production by monocytes, peritoneal macrophages, and alveolar macrophages upon activation by *S. aureus* are different (Kapetanovic et al. 2011). In contrast to monocytes and peritoneal macrophages, alveolar macrophages do not develop endotoxin tolerance (Smith et al. 1994; Fitting et al. 2004; Hoogerwerf et al. 2010). It was shown that the specific cellular and lung cytokine microenvironment was responsible for this peculiar behavior (Philippart et al. 2012). Murine alveolar macrophages, in contrast to peritoneal macrophages, do not express TLR9 (Suzuki et al. 2005), do not produce IL-10 in response to LPS (Salez et al. 2000), and the engagement of TLR3 and 4 in these cells does not lead to IFNβ production and IFNβ-dependent STAT1 activation (Punturieri et al. 2004). Finally, and most importantly, gene expression of bone marrow-derived macrophages and peritoneal macrophages

exposed to either IFNγ or IL-4 (two cytokines that are supposed to induced M1 and M2 macrophages, respectively) are not similar (Zhang et al. 2010a). Another convincing evidence has been reported by Chaudry' group in mice undergoing trauma-hemorrhage and sepsis. The authors showed that ex vivo LPS activation of spleen macrophages and blood monocytes led to a reduced production of TNF and IL-6 as compared to sham animals, whereas the production of these cytokines was enhanced in alveolar macrophages and Kupffer cells (Suzuki et al. 2006). Thus, it must be emphasized that differences exist between monocytes, macrophages, and their tissue subsets in terms of their response during sepsis (Fig. 14.3). While hyporeactivity of blood monocytes to ex vivo activation by endotoxin has been regularly observed in sepsis and SIRS, this is not the case for cells within the tissues. Moreover, the discovery of monocyte subsets, their differential expansion in sepsis, and their divergent response to TLR ligands adds a new complexity to the scenario (Fingerle et al. 1993; Blumenstein et al. 1997; Cros et al. 2010; Wong et al. 2011; Gautier et al. 2012; Shalova et al. 2012).

14.6 Endotoxin Tolerance as a Mechanism for Monocyte–Macrophage Dysfunction in Sepsis

Endotoxin tolerance is defined as the inability of an organism or its cells to respond to endotoxins as a result of a prior exposure to endotoxin. It is an adaptation to protect the host against excessive inflammation. Endotoxin tolerance was originally described by Paul Beeson in 1946, who repeatedly injected rabbits with typhoid vaccine to observe progressive reduction of fever induced by the vaccine (Foster and Medzhitov 2009). Similarly, mice injected with a sublethal dose of LPS protected them from subsequent challenge with a lethal dose of LPS (Cavaillon and Adib-Conquy 2006). Early studies indicated monocyte/macrophages as the principal cells responsible for the induction of ET in vivo. Since then, the induction of endotoxin tolerance in murine macrophages and human monocytes that have pre-exposure to sublethal dose of LPS has been demonstrated by several groups (Dobrovolskaia and Vogel 2002; Cavaillon and Adib-Conquy 2006; Foster et al. 2007; del Fresno et al. 2009). One of the cardinal features of in vitro endotoxin tolerance in these cells is the failure to express TNFα upon a second challenge with endotoxin. Other features include downregulation of HLA-DR and impaired antigen presentation (del Fresno et al. 2009). Upregulation of phagocytosis and genes involved in anti-microbial activity has also been reported for in vitro endotoxin tolerant murine macrophages and human monocytes (Foster et al. 2007; del Fresno et al. 2009; Fernandes et al. 2010). In line with this observation, in vivo endotoxin tolerance was shown to enhance bacterial clearance in mice (Murphey et al. 2008; Wheeler et al. 2008).

A recent study on in vitro endotoxin tolerance in human PBMCs and macrophages demonstrated the upregulation of genes related to wound healing (VEGFA, MMP9) and phagocytosis (MARCO, CD23) (Pena et al. 2011). Further, this study also showed upregulation of typical M2-specific markers CCL22, CCL24, CD163,

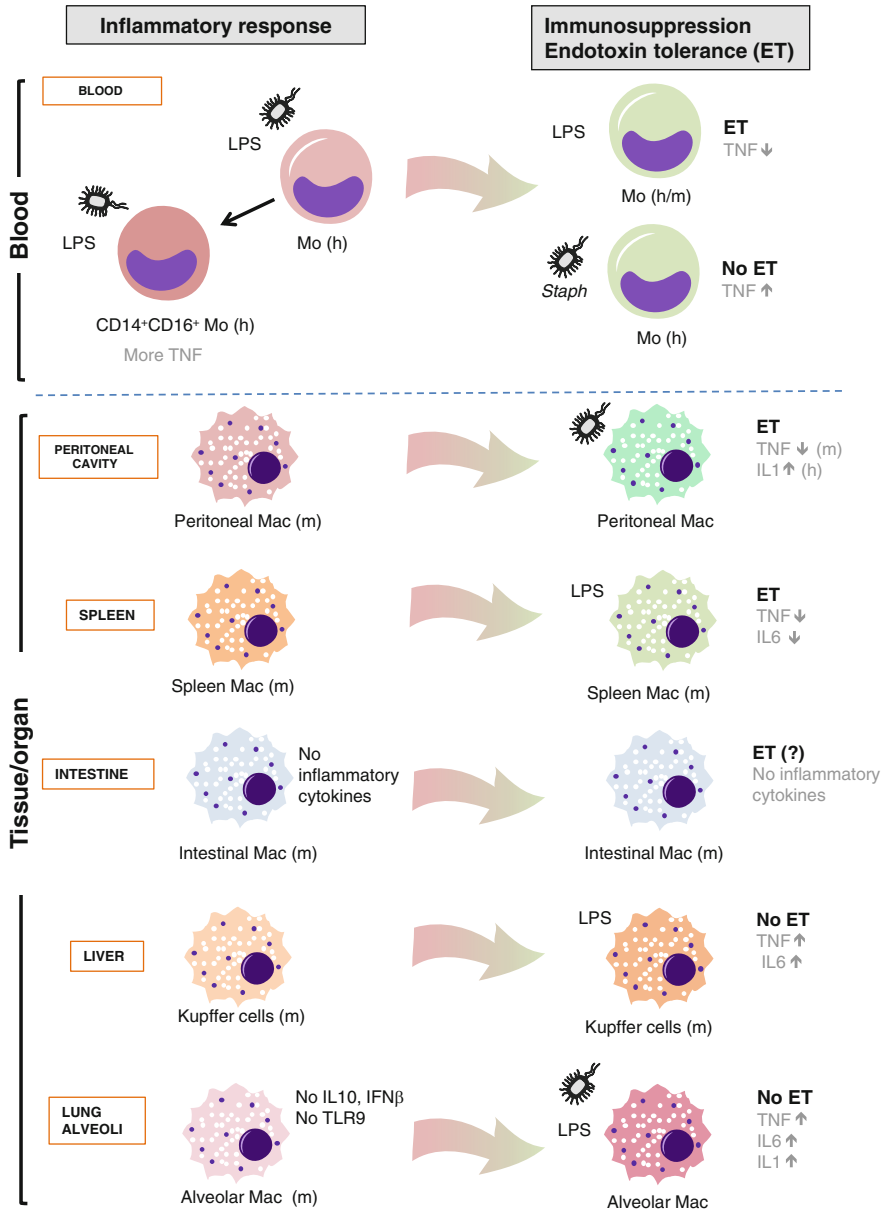

Fig. 14.3 Diversity of monocyte–macrophage response in blood and tissues. Schematic representation shows the diversity in the response of blood monocytes and different tissue macrophage populations in sepsis. *Left panel* depicts induction of an initial pro-inflammatory state in response to sepsis, while the *right panel* depicts their re-programming to an endotoxin tolerant (ET) state or not, upon re-challenge. The induction of ET not only varies with the cell type but also the type of ligands, e.g., LPS or *Staphylococcus* bacteria, as shown for monocytes. Also shown in this figure are subsets of monocytes ($CD14^+CD16^+$ monocytes) that expand during sepsis and their increased TNF response than $CD14^+$ monocytes. Findings specific to mouse or human are depicted by "m" or "h"

and CD206 in these cells. Similarly, endotoxin tolerant mouse murine macrophages show upregulation of Arg1, YM1, and Fizz1, suggesting an M2-like polarization of endotoxin tolerant monocyte–macrophages (Porta et al. 2009; Pena et al. 2011).

In essense, the various observations presented above suggest that monocyte–macrophages during endotoxin tolerance undergo genetic and functional reprogramming wherein pro-inflammatory features are downregulated, while anti-inflammatory, anti-microbial, and tissue remodeling properties are upregulated. Such a phenotype would be beneficial for protection of host during a sepsis. Indeed, many features of endotoxin tolerant monocyte/macrophage are mirrored by sepsis monocytes such as downregulation of HLA-DR and inability to upregulate inflammatory cytokines upon ex vivo LPS challenge (van Dissel et al. 1998; Escoll et al. 2003; del Fresno et al. 2009). Collectively, these observations indicate endotoxin tolerance as an important mechanism for orchestrating the immunosuppressive state of sepsis monocytes (see below).

14.6.1 Molecular Players in Endotoxin Tolerance and Sepsis

In the past 10 years, a number of reports have focused on the analysis of several molecules with potential implication in the control of sepsis development. As we have mentioned before, TLRs mediate host defense against microbial pathogens by eliciting production of inflammatory mediators and activating expression of MHC, adhesion, and co-stimulatory molecules which helps in the orchestration of a full-fledge immuno-inflammatory response to fight the pathogen. However, excessive TLR-driven inflammation during sepsis gives rise to endotoxin tolerance of monocyte–macrophage which lose their ability to respond to further endotoxin challenge, leading to increased risks of nosocomial infection, a common cause for mortality in most sepsis patients (Pachot et al. 2006; Hotchkiss et al. 2009). Thus, the main players that control sepsis progression could be the same as those that also control its refractory state or endotoxin tolerance. A large body of literature has identified various surface molecules, signaling intermediates, transcription factors and epigenetic changes in the induction of endotoxin tolerance in monocytes and macrophages. However, only a few have passed the "tests" in humans. Some of the main players are discussed below. A more detailed account on these molecules has been reviewed earlier (Biswas and Lopez-Collazo 2009).

14.6.2 Signaling Molecules and Negative Regulators

Studies in the last few years have identified several molecules that can negatively regulate TLR signaling in monocyte/macrophage and hence, possible candidates for endotoxin tolerance. These include molecules like *M*yeloid *d*ifferentiation *88 s*hort (MyD88s), IL-1R-associated kinase-M (IRAK-M), ST2, Suppressor of cytokine

signaling-1 and -3 (SOCS1, 3), Src homology 2-containing-inositol 5′-phosphatase (SHIP), and MAP kinase phosphatase-1 (MKP-1) (Xiong and Medvedev 2011). While knockout mice for many of these molecules show increased lethality to endotoxin shock in vivo, only a few of them showed promise in human studies. As for example, in a human in vivo endotoxemia model, the kinetics and expression of a panel of negative regulators (indicated above) were studied following intravenous injection of LPS in healthy volunteers (van 't Veer et al. 2007). However, only IRAK-M was found to be definitively associated with human LPS tolerance in this study.

IRAK-M belongs to the serine/threonine kinases, termed *I*nterleukin-1 *r*eceptor-*a*ssociated *k*inases (IRAKs). Four members of this family have been described to date, of which IRAK-1 and IRAK-4 are catalytically active (Cao et al. 1996; Li et al. 2002), whereas IRAK-2 and IRAK-M lack enzymatic activity due to single point mutations (Janssens and Beyaert 2003). IRAK-1 and IRAK-4 activation is a key step in triggering inflammatory response downstream of TLR/IL-1R while IRAK-M serves as a negative regulator of this pathway (Fig. 14.4). This pseudo-kinase is almost exclusively expressed in peripheral blood leukocytes, and is notably upregulated during the differentiation process of monocytic cell lines into

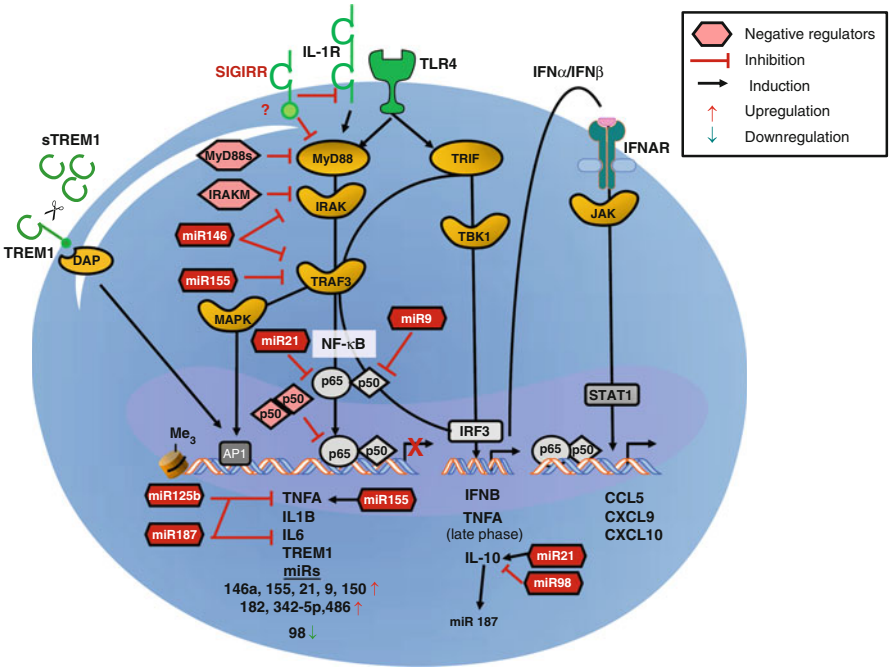

Fig. 14.4 Signaling and molecular mechanisms regulating monocyte–macrophage response in sepsis and endotoxin tolerance. The figure depicts a simplified scheme of some of the key signaling molecules, transcription factors, negative regulators, and miRNAs that regulate monocyte–macrophage response to gram-negative bacteria, LPS, or sepsis. Only those negative regulators that have been reported in human monocytes upon endotoxin challenge or sepsis are presented in the figure

macrophages (Kobayashi et al. 2002; Rosati and Martin 2002). Studies with IRAK-M-deficient mice provide strong genetic evidence for its negative regulatory role in TLR pathway, with implications in endotoxin tolerance and sepsis (Nakayama et al. 2004). In humans, early evidences showed upregulation of IRAK-M in the monocytes of septic patients (Escoll et al. 2003). Further, immunosuppression in human sepsis caused by *Burkholderia pseudomallei* was found associated with an upregulation of this pseudo-kinase (Dong et al. 2013). In this study, the grade of immunosuppression was correlated with mortality revealing that patients who died possessed higher IRAK-M mRNA levels on admission as compared to those who survived. In another study, genetic polymorphism in IRAK-M was associated with the susceptibility to sepsis (Lyn-Kew et al. 2010). In a study on pulmonary macrophages from mice undergoing *c*ecal *l*igation and *p*uncture (CLP)-mediated sepsis, transient impairment in LPS-induced cytokine responses and associated epigenetic changes were shown to temporally correlate with IRAK-M induction. Further analysis of IRAK-M$^{-/-}$ pulmonary macrophages indicated that systemic sepsis induces epigenetic silencing of cytokine gene expression in lung macrophages wherein IRAK-M seems to be a critical mediator of this response (Deng et al. 2006). IRAK-M's pivotal role in sepsis control has been demonstrated in different models (Lopez-Collazo et al. 2006). Collectively, these mice and human data clearly suggest that therapies based on IRAK-M expression control could be an important means of regulating the function of monocytes and macrophages in sepsis.

In addition to above, the expression of negative regulators like *Toll i*nteracting *p*rotein (Tollip), SOCS1, MyD88s, and *S*ingle *i*mmuno*g*lobulin *i*nterleukin-1 *receptor-related* molecule (SIGIRR) have also been investigated in sepsis monocytes (Adib-Conquy et al. 2006). Sepsis monocytes showed significant upregulation of MyD88s and SIGIRR but not Tollip or SOCS1, as compared with healthy controls (Fig. 14.4). However, these analyses were done in parallel with resuscitated patients after cardiac arrest and ex vivo stimulation with different TLR ligands and heat-killed bacteria. Importantly, these gave distinctly different outcomes suggesting that the final response is largely dependent on the type of signaling pathway that is triggered.

SHIP is another negative regulator of TLR pathway. Mice deficient in SHIP have been shown to be protected from endotoxin shock (Sly et al. 2004). However, study in human endotoxemia model did not show any upregulation of this molecule in mononuclear cells (van 't Veer et al. 2007). A more detailed analysis of SHIP in humans will possibly explain the reason for this deviation or the fact that the function of SHIP in mouse and human is different.

*T*riggering *r*eceptor *e*xpressed on *m*yeloid cells-*1* (TREM-1) is a receptor member of the immunoglobulin superfamily expressed on the cell surface of mature monocyte–macrophages and granulocytes. LPS and other microbial products induce upregulation of cell surface-localized TREM-1 and the release of its soluble form, sTREM-1 by human monocytes (Fig. 14.4). This latter form (sTREM-1) is generated by a metalloproteinase shedding of the TREM-1 ectodomain through proteolytic cleavage of its long juxtamembrane linker (Gomez-Pina et al. 2007). TREM-1 is involved in amplifying inflammatory response initiated by the TLR. The in vivo functional significance of TREM-1 in amplifying such inflammatory

response is demonstrated by mice studies where blockage of TREM1 protected mice from LPS-induced septic shock (Gibot 2005). Similarly, synthetic peptide mimicking sTREM1 could also attenuate inflammatory cytokine production by human monocytes in vitro. In vivo administration of LPS to healthy humans upregulated TREM1 expression in monocytes, but not in granulocytes (Knapp et al. 2004). TREM1 expression on monocytes as well as sTREM1 levels has been reported to be upregulated in sepsis patients (Gibot 2005; Oku et al. 2013) (Fig. 14.4). In this regard, sTREM-1 may be useful to evaluate disease severity and the outcome of sepsis patients (Jeong et al. 2012). sTREM-1 measurement was shown to predict the development of sepsis-associated acute kidney injury (Derive and Gibot 2011). Accordingly, urinary soluble TREM-1 levels were significantly elevated in this kind of patients. In addition, when survivors were compared to non-survivors, only sTREM-1 level [but not other biomarkers like *C-r*eactive *p*rotein (CRP) and Procalcitonin] remained significantly higher in non-survivors until death (Derive and Gibot 2011). These data indicate TREM-1 as a regulator and potential biomarker of sepsis progression. Interestingly, while engaging membrane TREM1 can amplify inflammatory signaling, mimics to sTREM1 can attenuate such response, which raises the question as to whether TREM1/sTREM1 axis may be important for regulating the plasticity of monocytes/macrophage from the inflammatory to refractory phenotype, during sepsis. Further studies should be undertaken to clarify this point.

14.6.3 Transcription Factors

Nuclear Factor-kappa B (NF-κB) is an integral transcription factor that regulated the transcription of inflammatory mediators downstream of the TLR pathway. During TLR signaling, signal-dependent degradation of the Inhibitor of Kappa B (I-κB) releases the activatory p65/p50 NF-κB heterodimer to trigger inflammatory gene transcription (e.g., TNFA, IL8, IL6 etc.) (Fig. 14.4). However, during endotoxin tolerance, human monocytes and mouse macrophages have been shown to accumulate the p50/p50 homodimer, which inhibits the binding of p65/p50 heterodimer to inflammatory gene promoters (such as TNF) and prevents their transcription (Fig. 14.4). Supporting this, experiments in p50 NF-κB$^{-/-}$ macrophage showed lack of LPS tolerance and the failure of long-term LPS pretreatment to block TNF mRNA in these cells (Bohuslav et al. 1998). In human monocytes, LPS tolerance induced upregulation p50/p50 homodimer, inhibition of TNF expression but increase in IL-10 (Ziegler-Heitbrock 1995). Further studies using p50 NF-κB$^{-/-}$ mice revealed that IL-10-induced inhibition of inflammatory genes in macrophages during LPS tolerance is possibly mediated by the induction of p50/p50 NF-κB homodimers (Driessler et al. 2004). Extending these observations, blood mononuclear cells from patients with severe sepsis or trauma are reported to show a general reduction of p65/p50 NF-κB heterodimers as compared to the control group (Adib-Conquy et al. 2000). Comparison of NF-κB members in survivor versus

non-survivors revealed a global downregulation of NF-κB in survivors whereas the presence of large amounts of the p50/p50 NF-κB in the non-survivors of sepsis. Moreover, a reverse correlation between plasma IL-10 levels and the p65p50/p50p50 ratio in the mononuclear cells was found in the non-survivors after in vitro LPS stimulation (Adib-Conquy et al. 2000).

Distinct from the downregulation of inflammatory genes (associated with the M1 macrophage phenotype) by p50/p50 NF-κB homodimers, p50 NF-κB was recently implicated in inducing the expression of M2 genes in murine macrophages during endotoxin tolerance (Porta et al. 2009). Together, these evidences suggest p50 NF-κB as a crucial regulator of monocyte–macrophage function in endotoxin tolerance and sepsis. Other NF-κB members like RelB have also been implicated in the induction of endotoxin tolerance in monocytes during sepsis (Yoza et al. 2006).

14.6.4 Epigenetic Regulation

Several evidences suggest epigenetic changes to contribute to gene re-programming associated with endotoxin tolerance with implications in sepsis. The epigenetic control can either involve direct chromatin remodeling processes or post-translational control by microRNAs (miRNAs). Chromatin remodeling essentially involves histone modifications which affect the coiling of the chromatin and hence its accessibility to transcription. Histone modifications such as deacetylation or methylation cause gene silencing, while its acetylation or demethylation of histones triggers transcription of target genes (Wilson 2008). In vitro studies on LPS tolerance in murine macrophages have defined two distinct groups of genes that are modulated in an opposite fashion as a result of distinct epigenetic mechanisms (Foster et al. 2007). Promoters of inflammatory genes (e.g. IL1B, IL6) which were downregulated during LPS tolerance, remained histone methylated during the first and second LPS challenge, indicating an inhibition of transcription (Fig. 14.4). In contrast, promoters of anti-microbial genes (e.g. FPR1, LCN2) that were upregulated during LPS tolerance, underwent histone methylation during the first LPS exposure, but were re-acetylated upon the second LPS challenge, poising them for further transcription. Similarly, several other reports also demonstrated epigenetic mechanisms for silencing of pro-inflammatory genes in human monocytic cells during endotoxin tolerance. For example, RelB induced histone dimethylation at the IL1B promoter which inhibited p65 TNF-κB transactivation and hence transcription. On the same lines, gene silencing of TNF and IL1B was shown to be mediated by the binding of high-mobility group box 1 proteins (HMGB1) and histone H1 linker at their promoters (El Gazzar et al. 2009). Moreover, gene products induced by LPS could act as epigenetic regulators for gene expression during subsequent LPS challenge. For example, the LPS-induced histone demethylase, JMJD3 regulates chromatin remodeling necessary for the expression of M2 macrophage-specific genes (Satoh et al. 2010). This may suggest a potential mechanism for switching of inflammatory macrophages to an M2 phenotype during endotoxin tolerance.

Another new class of epigenetic regulation is provided by the microRNAs (miRNAs). miRNAs are a small class of non-protein-coding RNAs (21–23 nucleotides) that regulate the expression of target mRNAs post-transcriptionally (See Chap. 27, by Locati and colleagues for details). These molecules are associated with a growing number of biological processes and the expression signatures of miRNAs may serve as diagnostic and prognostic markers for various diseases. During sepsis, several miRNAs have been found in monocyte/macrophages including miRNA-150, miRNA-182, miRNA-342-5p, miRNA-486, and miRNA-146a/b (Mitchell et al. 2008; McDonald et al. 2011; Wang et al. 2013). Interestingly, some authors have demonstrated that miRNA-146a could be a potential marker to differentiate sepsis from non-sepsis-SIRS (Rossato et al. 2012; Quinn et al. 2013). In particular, this miRNA can play a key role in endotoxin tolerance by negative regulation of TLR signaling via downregulation of IRAK-1 and TRAF6, and the inhibition of TNF expression (Savva et al. 2011) (Fig. 14.4). Other miRs such as miRNA-155 and miRNA-125b are reported to inhibit LPS/TLR4 signaling and/or TNF expression, indicating a potential role in endotoxin tolerance (O'Connell et al. 2007; Tili et al. 2007). In contrast to targeting signaling molecules and inflammatory cytokines, miRNAs such as miR-98 can negatively regulate the expression of anti-inflammatory cytokines like IL-10 post-transcriptionally (Liu et al. 2011). However, miR-98 is downregulated upon LPS stimulation and thereby suggested to play a role in the induction of endotoxin tolerance via IL-10 expression in macrophages. On the same lines, miRNA-21 is expressed by LPS-activated macrophages and tunes-down inflammation via IL-10 induction (Sheedy et al. 2010). IL-10 was recently described to induce the expression of miRNA-187 at late times, following LPS treatment in human monocytes (Rossato et al. 2012). This miRNA can downregulate inflammation by targeting TNF, NFKBIZ, and other cytokines like IL-12 and IL-6. It would be interesting to see whether these miRNAs are also modulated under sepsis and, thereby contribute to the monocyte–macrophage re-programming.

14.7 From the Bench to Bedside

14.7.1 Biomarkers in Sepsis

Because the response of the body to blood infection can be very similar to its reaction to non-infectious "challenges" (e.g. trauma), sepsis is difficult to characterize. Several biomarkers have been tested: but to date, not a single one distinguishes sepsis from other systemic inflammatory diseases, adequately. This is a challenge for both basic and clinical research.

Besides the well-known procalcitronin (PCT), two molecules related to monocyte/macrophage have been the focus of some studies. On one hand, as we have mentioned before the soluble isoform of TREM-1 has been found to significantly increase in septic patients who ended up dying (Derive and Gibot 2011; Jeong et al. 2012; Oku et al. 2013). On the other hand, levels of soluble urokinase-type plasminogen

activator receptor (suPAR) distinguish dying patients from those who have sepsis and pneumonia, but would survive (Hotchkiss and Karl 2003). This molecule too could become a potential biomarker and is expressed as a surface protein on neutrophils and monocyte/macrophages. While future research may possibly reveal other potential biomarkers of monocyte/macrophage origin, taking into account all the data published, there is probably not a single molecule to mark sepsis but, a set of them. A combination of biomarkers would be more effective than any single biomarker.

14.7.2 Therapies and the Future

It is well established that patients with sepsis develop some clinical features including fever, shock, and respiratory failure. This is due to an uncontrolled inflammatory response. In this regard, the discovery that level of several cytokines increase during sepsis, and when injected into animals these reproduce clinical features of sepsis, prompted physicians to understand sepsis as a "Cytokine storm." However, the results of an important number of clinical trials have shown that TNFα and IL-1 antagonists, TLR blockers, and endotoxin inhibitors do not report patient benefits (Meisel et al. 2009). Further, accumulated data from clinical trials and clinical experience suggest that immunomodulatory therapies in sepsis should be personalized on the basis of each particular development. In this line, it has been observed that a septic patient who has entered the immunosuppressive phase (they show a persistent low level of HLA-DR) is susceptible to be treated with granulocyte macrophage colony-stimulating factor (GM-CSF). The results demonstrated that these patients restored HLA-DR expression and their intensive care assistance was reduced (Huang et al. 2009). A recent meta-analysis on GM-CSF and G-CSF therapy in sepsis patients failed to demonstrate any benefit in terms of mortality, but showed an increased incidence of reversal rate from infection (Bo et al. 2011). Another putative target is the negative co-stimulatory molecule called PD-1. Signaling through PD-1 inhibits T-cell proliferation as well as their cytokine production. During sepsis, monocyte/macrophages could overexpress PD-1 ligand on their cell surface as well as septic T-cells could also show high levels of PD-1. Their interactions induce a clear immunosuppression. Several authors have demonstrated that blockage of the PD-1 signaling improved survival in animal models of sepsis (Brahmamdam et al. 2010; Zhang et al. 2010b). These indicate that PD-1 blockers could work in those patients whose flow cytometric quantification of circulating cell expression of PD-1/PD-1 ligand is high. Thus, all these data suggest that immunotherapy has wide-ranging advantageous effects in sepsis, but it must be personalized.

Most importantly, organ failure more than immune failure can be rendered responsible of the death of sepsis patients. Organ failure is mainly due to an increased apoptosis of endothelial cells and epithelial cells in tissues and an alteration of tight junction. Therefore, the challenge for new therapies would be to reduce the apoptosis of these cells and eventually that of lymphocytes and dendritic cells, while reducing the enhanced resistance to apoptosis displayed by neutrophils.

For therapies aimed to modulate the immune system, the aim should be to boost the immune status of blood monocytes, but downregulate the hyperactivity displayed by macrophages within the tissues. Such an approach has already been obtained in experimental models with androstenediol (Suzuki et al. 2006). Accordingly, there is hope to improve the outcome of sepsis patients if one avoids a too simplistic analysis of the inflammatory process and integrates the fact that mononuclear phagocytes may not behave similarly in the different compartments.

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Chapter 15
Adaptive Characteristics of Innate Immune Responses in Macrophages

Mihai G. Netea and Alberto Mantovani

15.1 Macrophages Are Cells Crucial for Both Innate and Adaptive Immune Responses

Macrophages are a central component of antimicrobial host defense, described as crucial for both innate immune mechanisms and adaptive immunity (Gordon and Mantovani 2011). The dichotomy between the immediate antimicrobial responses seen as non-specific, and the late-onset specific T- and B-cell responses, has been driven our understanding of host defense for more than half a century. Innate immunity reacts instantly upon an encounter with a pathogen, but has been viewed as non-specific and incapable of building an immunological memory. In contrast, adaptive immune responses can specifically recognize pathogenic microorganisms and build memory capable of protection against reinfection. Macrophages are involved in both these two responses: on the one hand macrophages have the capacity to phagocytose and kill microorganisms in a non-specific fashion, to release proinflammatory mediators that drive inflammation, but on the other hand they can also present antigens and initiate and modulate the specific T-cell responses through expression of co-stimulatory molecules and specific cytokines (Taylor et al. 2005).

The last decade has dramatically changed the dogma of innate immunity being non-specific, through the discovery of pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RigI-helicases. These receptors are expressed either on the surface of

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macrophages (and other immune cells) or intracellularly to allow specific recognition of conserved structures of different classes of microorganisms (Takeuchi and Akira 2010). PRRs allow thus semi-specific recognition of different types of microorganisms, though not individual microbial species. The widespread conviction that innate immunity in general, and macrophages in particular, cannot adapt to a previous encounter with a pathogen, and is incapable of building the memory of a previous infection, has not been however contested.

15.2 Immunological Memory Within Innate Immune Responses

An unbiased survey of immunological literature reveals however important clues that innate immunity is able to display adaptive features and that these mechanisms can offer protection to reinfection, independently of the classic adaptive immune memory (Bowdish et al. 2007; Netea et al. 2011). The adaptive characteristics of innate immunity, sometimes also called "trained immunity" (Netea et al. 2011), are manifested as protection against reinfection by the same or different pathogens in organisms lacking adaptive immune responses such as plants (Durrant and Dong 2004), invertebrates (Pham et al. 2007; Kurtz 2005), or mammals lacking functional T- and B-cells (Sun et al. 2009; Paust et al. 2010; Quintin et al. 2012) (Table 15.1). The discovery of memory characteristics of prototypic innate immune cells such as natural killer (NK) cells or monocytes has been viewed as a paradigm shift in immunity (Bowdish et al. 2007; Vivier et al. 2011). In this context, several studies have demonstrated that macrophages have a crucial role in the T/B-cell independent protection mechanisms against reinfection (Quintin et al. 2012; Bistoni et al. 1986).

15.3 Macrophage Priming, Activation, and Tolerance as Adaptive Responses

Since the late 1960s it has been known that following exposure to microbes or to microbial components, mononuclear phagocytes show increased effector functions as revealed by their augmented microbicidal and tumoricidal activity (Mackaness 1969; Adams and Hamilton 1984; Evans and Alexander 1970) (Fig. 15.1). Mirroring the increased effector function, exposure to microbial agents in vivo was found to be associated with increased non-specific resistance to pathogens (Kleinnijenhuis et al. 2012). Classic activation of macrophages requires hours and the enhanced effector

Table 15.1 Examples of memory of innate immunity in plants, invertebrates, and mammals

Organism	Experimental model	Biological effect	Specificity	Mechanism
Plants—"systemic acquired resistance"				
Large variety of plants	Viruses, bacteria, fungi	Protection against reinfection	Variable	Salicylic acid Epigenetic mechanisms
Adaptive immune responses in non-vertebrates				
Beetle	LPS, or bacterial prechallenge	Protection against reinfection	–	Trans-generational priming
Drosophila	*S. pneumonia* *B. bassiana* *S. marcescens*	Protection	+	Serine protease CG33462
Anopheles gambiae	Midgut flora	Protection against *Plasmodium*	+	Toll-dependent hematocyte-differentiation factor
Adaptive characteristics of innate immunity in mammals				
Mice	*C. albicans* BCG	Protection against candidiasis	–	Monocyte epigenetic reprogramming
Mice	Murine CMV Hypersensitization	NK-dependent	+	Ly49^{+} NK cells Hepatic CXCR6^{+} NK cells
Humans	BCG vaccination	Protection to non-related infections	–	Monocyte reprogramming

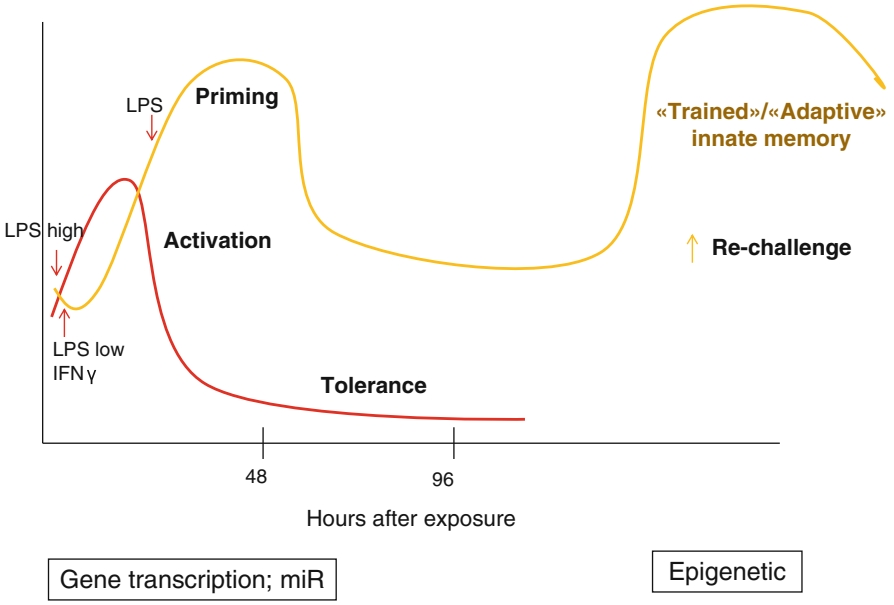

Fig. 15.1 Priming, activation, and tolerance as adaptive macrophage responses. A schematic representation of the possible responses of macrophages to a novel stimulus (e.g., LPS). While LPS causes macrophage activation followed by tolerance, low doses of LPS or IFNγ prime macrophages for enhanced innate immune responses, followed by tolerance. Microbial signals can give rise to long-term changes in macrophage function ("memory")

functions are transient. It was subsequently discovered that co-exposure to selected cytokines (the prototype of which is interferon-γ) resulted in a dramatic increase in the responsiveness of macrophages to microbial moieties (Adams and Hamilton 1984; Nathan et al. 1984) (Fig. 15.1). After exposure to IFNγ, macrophages retain a primed state for several hours, with a dramatic augmentation of responsiveness for instance to LPS in terms of toxic mediators (NO), cytokines (TNFα and IL-12), chemokines, or co-stimulatory molecules (Adams and Hamilton 1984; Nathan et al. 1984; Meltzer et al. 1982). Priming of human monocytes is in part sustained by increased expression of PRRs (TLR4 and MD2) and transducers (MyD88) (Bosisio et al. 2002). The discovery of alternative forms of macrophage activation, originally by IL-4 (Stein et al. 1992) has opened a new perspective on the plasticity of macrophages and their activation states (Sica and Mantovani 2012). Moreover, transcriptional profiling (e.g., in Martinez et al. (2006)) has revealed the complexity of gene regulation during "activation" and paved the way to the dissection of underlying mechanisms (Sica and Mantovani 2012; Monticelli and Natoli 2013).

Deactivation can also occur as a consequence of microbial encounter (Fig. 15.1). Microbial signals (e.g. LPS) under appropriate conditions can result in tolerance, i.e., in hyporesponsiveness to a subsequent challenge at the macrophage and organism level. The immunosuppressive phenotype observed in late sepsis is likely a reflection of tolerance to LPS and/or other bacterial ligands. Tolerance is generally viewed as a defense strategy to limit inflammation-caused tissue damage (Medzhitov et al. 2012). The definition of tolerance is actually misleading as is "activation." Analysis of the macrophages transcriptome has revealed that LPS tolerance is in fact a manifestation of reorientation of macrophage functions. The transcriptional profile of LPS-tolerant macrophages has some similarity to that expressed by alternatively activated M2-polarized macrophages (Akey 2009; Biswas and Lopez-Collazo 2009). Tolerant macrophages have an elevated expression of interleukin-10 (IL-10), arginase I, and the chemokines CCL17 and CCL22. Thus, what has long been viewed as endotoxin tolerance is not simple unresponsiveness: it represents an adaptive response of macrophages with reorientation to an immunoregulatory phenotype.

The interaction with microbial components not only affects, but also profoundly alters the receptor repertoire expressed by cells of the monocyte–macrophage lineage (Bowdish et al. 2007; Willment et al. 2003). It has been shown that microbes and cytokines change the levels of dectin-1 and of the scavenger receptor MARCO and hence modify subsequent responses to their ligands (Bowdish et al. 2007; Willment et al. 2003).

In addition to changing the levels and repertoire of macrophage surface receptors, inflammatory signals also induce production of fluid-phase patterns recognition molecules (PRMs). These molecules constitute the humoral arm of innate immunity together with complement (Ricklin and Lambris 2013; Hajishengallis and Chavakis 2013), and they function as ancestors of antibodies ("ante-antibodies") (Bottazzi et al. 2010). Myelomonocytic cells are the source of a vast range of fluid-phase PRMs. These include collectins (e.g., mannose binding lectin, MBL), ficolins (e.g., L-, H-,

and M-ficolin), and pentraxins (e.g., Pentraxin 3, PTX3) (Bottazzi et al. 2010). PTX3 has served as a paradigm for the mode of action of the interplay between the cellular and the humoral arm of innate immunity (Jeannin et al. 2005). This "long" pentraxin is produced in a gene-expression dependent way by mononuclear phagocytes. In addition, it is stored in granular compartments in neutrophils. PTX3 is an essential component of resistance to selected pathogens such as *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. The effector mechanisms utilized by PTX3 include recognition and binding to microbial components, activation, and regulation of the complement cascade and opsonization-mediated destruction of pathogens (Ricklin and Lambris 2013; Bottazzi et al. 2010; Jeannin et al. 2005). In addition, PTX3 binds P-selectin and reduces neutrophil recruitment to sites of inflammation dampening inflammation (Hajishengallis and Chavakis 2013; Deban et al. 2010). Thus, PTX3 and other soluble PRMs produced by phagocytes serve as an amplification and regulatory loop in the phagocyte-mediated resistance and tissue damage.

The regulation of macrophage function in the lungs provides a good example of long-term conditioning of macrophage responsiveness (Naessens et al. 2012; Didierlaurent et al. 2008). After resolution of viral infections (influenza, respiratory syncytial virus), long-lasting lung macrophage desensitization was observed (Didierlaurent et al. 2008). Interestingly, unresponsiveness was mediated by TLR4 desensitization, a finding reminiscent of previous in vitro and in vivo studies (Kleinnijenhuis et al. 2012; Bosisio et al. 2002). On the other hand, after exposure to allergic bronchial inflammation, alveolar macrophages show increased inflammatory responses to TLR ligands, with acquisition of the capacity to produce IFN-β (Naessens et al. 2012). Thus, infectious or inflammatory conditions may imprint lung macrophages for attenuated or increased responsiveness. Defining the molecular basis of adaptive responses of lung macrophages may have implications for better understanding of the pathogenesis of diverse clinical conditions.

15.4 Molecular Mechanisms Driving the Adaptive Characteristics of Innate Immunity

The importance of immune adaptation as an evolutionarily driven property of complex defense systems is underlined by the discovery of an independently developed second type of adaptive immune system in jawless vertebrates, based on variable lymphocyte receptors (Boehm et al. 2012). Complementary to these phylogenetic arguments, studies investigating the ontology of the immune system have demonstrated that TLR-induced maturation of innate immunity (as assessed by cytokine profiles) is an adaptive feature of mammalian host defense that is designed to reduce the impact and severity of subsequent infections (Levy 2007; Philbin et al. 2012).

Building on these evolutionary arguments, an overview of the rich body of research documenting adaptive traits of innate immunity in plants and invertebrates provides a first set of strong arguments for innate immune memory and the molecular

mechanisms that drives it (Netea et al. 2011; Kurtz 2005). Systemic acquired resistance (SAR) has been described as the central process providing protection against reinfection in plants. The study of the biochemical mechanisms mediating SAR has provided the first clues that epigenetic processes are crucial for innate immune memory (van den Burg and Takken 2009; Conrath 2011). Interestingly, epigenetic changes have been recently shown to be able to provide transgenerational transmission of resistance, with the acetylation of H3K9 being central for this process (Slaughter et al. 2012). The epigenetic programs induced in plants during SAR represent a first clue regarding the mechanisms that induce long-term changes in the functional phenotype of innate immune cells.

How the function of macrophages is modulated by microbial stimuli is an important aspect in determining host defense. Recent studies have reported that the dose of LPS determines whether tolerance (by high LPS concentrations) or priming (by ultra-low LPS concentrations) is attained when macrophages are stimulated (Maitra et al. 2012). Epigenetic mechanisms were shown to be central to the process of LPS tolerance (Foster et al. 2007). Important new insights by a recent study shed more light on these processes during cell stimulation: several types of epigenetic modifications involving both histone methylation and acetylation are induced by LPS in a cell type- and stimulus-specific manner (Ostuni et al. 2013). However, while most of these markers fade in time, histone methylation at H3K4me1 in so-called "latent enhancers" remains active as an epigenetic marker of this process, conferring long-term immunological memory (Ostuni et al. 2013). The importance of histone methylation for the epigenetic reprogramming of monocytes at the H3K4 level has been also very recently demonstrated following exposure to β-glucans, leading to an increased response to a secondary stimulation (Quintin et al. 2012) (Fig. 15.2). The trained innate immunity induced in vivo through epigenetic reprogramming results in protection of T/B-cell-deficient *Scid* and *Rag1*$^{-/-}$ mice against lethal systemic candidiasis, underlining the therapeutic potential of the adaptive characteristics induced in macrophages (Quintin et al. 2012). An important aspect observed in these studies is the lack of specificity conferred by trained immunity, in which protection is given against not only the original microorganism, but additional microorganisms as well.

To conclude, these data suggest a picture in which the innate immune system is characterized by adaptive features, and can be trained to provide a partial protection against infection independent of the classical T/B-cell adaptive immunity. Functional reprogramming, especially through epigenetically mediated mechanisms, mediates these effects.

15.5 Consequences for Human Diseases

What are the consequences of the capacity of innate immunity to build immunological memory? Despite the data demonstrating the role of innate immune memory in plants and invertebrates, one may speculate that in mammals the biological relevance of the adaptive characteristics of innate immunity was lost during evolution, because

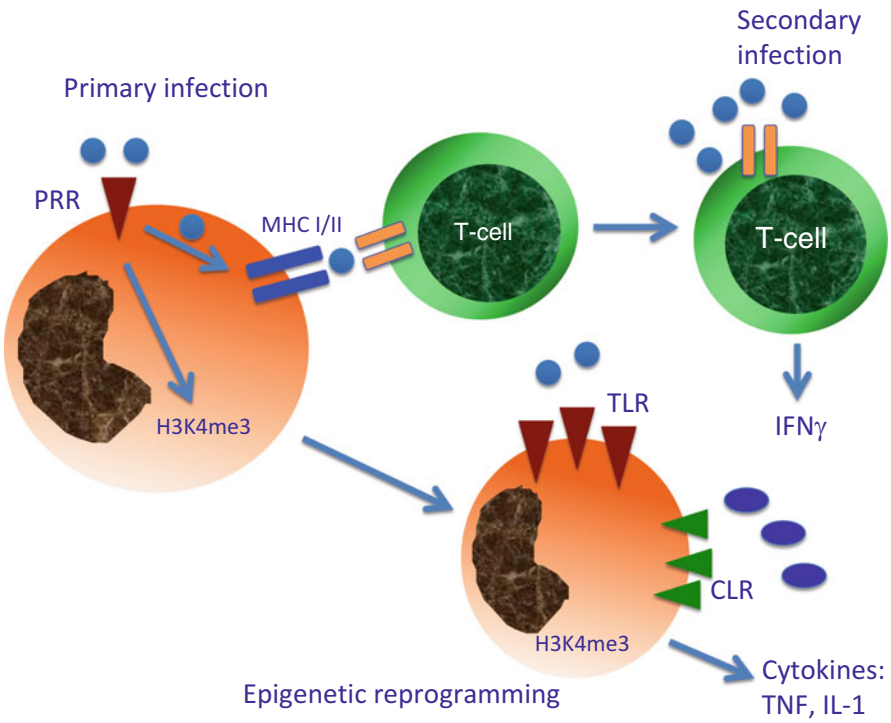

Fig. 15.2 Macrophages play a double role for inducing immunological memory following an infectious insult: on the one hand they initiate adaptive immune responses, and on the other hand they undergo epigenetic reprogramming to respond with an increased array of PRR expression and inflammatory cytokine production to a secondary infection ("trained innate immunity")

classical T/B-cell dependent adaptive immunity conferred the specificity needed during reinfection**.** However, there are several arguments to believe that trained immunity remains an important component of host defense. For example, the commensalism of certain microorganisms with the mammalian host may mirror their function in inducing non-specific immune protection. It is tempting to hypothesize that this may be the case for *Candida albicans*, a very common colonizer of human skin and mucosa that strongly induces trained immunity (Quintin et al. 2012), and this has also been suggested for the herpesvirus latency (Barton et al. 2007).

In addition, inducing an adaptive response of innate immunity is likely to have important therapeutic potential. Vaccines such as BCG have been shown to protect non-specifically animals against fungal sepsis (van 't Wout et al. 1992), and BCG can non-specifically protect mice against influenza infection (Spencer et al. 1977). If this effect were observed in humans, this would represent an unorthodox approach to vaccination in situations when specific vaccines are not (yet) available, such as in a global pandemic with a novel influenza virus. Indeed, non-specific protective effects against non-mycobacterial infections have been reported to be both very quick and last for years when children are vaccinated with live vaccines such as

BCG or measles vaccine (Aaby et al. 2011; Roth et al. 2006), and the number and strength of these epidemiologic data sets strongly advocate for a sustained effort to understand trained immunity and its potential therapeutic effects. More in general the results and concepts discussed here call for monitoring of the impact of new adjuvants on innate immunity and resistance to unrelated microorganisms. The challenge for the coming years is thus to deconstruct the molecular mechanisms mediating the adaptive characteristics of macrophages and other innate immune cell, as well as to harness these protective effects in clinical practice. Inducing trained innate immunity may be advantageous in various clinical conditions, from large-scale vaccination programs in settings of high infectious pressure in developing countries, to vaccination of patients lacking a functional immune system (e.g., HIV patients), to the reversal of immunoparalysis in sepsis.

In conclusion, the adaptive characteristics of innate immunity in general, and of macrophages in particular, have emerged as an important new property of innate host defense mechanisms. Its study in the coming years promises to become an area of very active immunological research, with a direct impact on our understanding of immune responses and on the design of novel immunotherapies.

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Chapter 16
African Trypanosomiasis as Paradigm for Involvement of the Mononuclear Phagocyte System in Pathogenicity During Parasite Infection

Benoit Stijlemans, Alain Beschin, Patrick De Baetselier, and Geert Raes

16.1 Introduction

African trypanosomiasis is a parasitic disease of medical and veterinary importance that has adversely influenced the economic development of sub-Saharan Africa. The causative agents, the trypanosomes, are hemoflagellated blood-borne unicellular protozoan parasites that are transmitted through the bite of their vector (i.e., the tsetse fly, *glossina spp.*) and cause fatal diseases in mammals, commonly called sleeping sickness in humans (HAT, Human African Trypanosomiasis) or Nagana in domestic livestock. The World Health Organization estimates that approximately 300,000 new cases occur within 60 million people living in the risk areas (i.e., the "tsetse" belt) leading to 50,000 deaths annually (Steverding 2008). In addition, the impact of HAT trypanosomiasis measured in terms of DALY (Disability-Adjusted-Life-Years) mounts up to 1,570 days (Mathers et al. 2007; Fevre et al. 2008). Using this measurement, the disease impact is as high as the impact of *Schistosomiasis* and *Leishmaniasis*, and far greater than the impact of Chagas' disease (i.e., American trypanosomiasis). Among the large variety of trypanosome species found, only two species, i.e. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*, are infectious to man (Malvy and Chappuis 2011). These human infecting trypanosomes have evolved a mechanism to resist lysis by innate immune factors present in the blood circulation, i.e. a member of the apolipoprotein L family termed apoL1 (Vanhamme et al. 2003; Vanhollebeke and Pays 2010) and haptoglobin-related protein (Hpr) (Vanhamme et al. 2003; Pays et al. 2006; Perez-Morga et al. 2005; Vanhollebeke et al. 2008; Dejesus et al. 2013). Although none of the other trypanosome species present in Africa cause diseases in humans, livestock infections can

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have indirect devastating effects on public health. Indeed, animal trypanosomiasis (i.e., Nagana), mainly caused by *T. congolense* and to a lesser extent by *T. b. brucei* and *T. vivax*, forms a major constraint on cattle production. Hence, Nagana has a great impact on the nutrition of millions of people living in the most highly endemic areas, and on the agriculture economics of their countries. Hereby, the annual economic cost of animal trypanosomiasis is estimated about US$4 billion (Ilemobade 2009).

As pure extracellular parasites, African trypanosomes are continuously confronted with the multiple components of the host's immune system, ranging from innate to adaptive immune defenses. Hence, co-evolution has resulted in the appearance of well-balanced growth regulating systems, allowing the parasites to survive sufficiently long without killing its mammalian host, in order to ensure an effective transmission of the species. To this end, these parasites have developed efficient immune evasion mechanisms (Namangala 2011). Firstly, trypanosomes are covered by about 10^7 identical densely packed molecules of an exposed immunodominant variant-specific surface glycoprotein (VSG) coat. Antigenic variation of this VSG coat allows escape from antibody (Ab)-mediated elimination by the immune system. Secondly, the hosts' capacity to mount an efficient B-cell mediated immune response and to maintain its immunological memory is undermined due to the loss in spleen architecture as well as a drastic disappearance of marginal zone B cells and B-cell depletion or hindered reactivation (Bockstal et al. 2011). Importantly, the destruction of the B-cell memory compartment appears to affect not only anti-trypanosome responses but also the immunological memory in general [reviewed by Magez et al. (Magez et al. 2010) and La Greca et al. (La Greca and Magez 2011)]. Thirdly, a profound suppression of T-cell responses occurs following infection by trypanosomes, which lowers the host's resistance to other infections and results in often fatal secondary diseases (Mansfield and Paulnock 2005). It should also be remarked that immunological lesions to tissues, including the spleen, lymph nodes, myocardium, brain, kidneys, and liver are common in natural African trypanosomiasis, which prevent the normal functioning of the affected organ and thereby compromise both the host survival and the parasite transmission (Ikede and Losos 1972). Indeed, morbidity and mortality in trypanosome-infected cattle are primarily due to self-inflicted damage by disproportional immune and/or innate responses (Naessens 2006). It is therefore important to identify the cellular and molecular players governing the delicate balance between eradication of the parasite by the hosts' immune response (i.e., parasite control) and limitation of the undesirable secondary effects (i.e., pathogenicity development).

Due to practical considerations as well as high costs and difficulties associated with bovine challenge experiments, the easier accessible mouse models have become an exquisite alternative to study the underlying mechanisms involved in trypanosomiasis-associated immunopathogenicity (Naessens 2006). Most of the studies in mice so far were performed with two African trypanosome species: *T. congolense* and *T. b. brucei*, whereby the first one resides strictly in the blood vessels and capillaries, while the latter travels also in the blood but principally

resides in the lymph and peripheral organs (Naessens 2006). This might also explain the different results obtained with both models with respect to parasite control mechanisms and host immune responses mounted (see later). However, both parasite models share two prominent pathogenic features in common, i.e. liver pathogenicity and anemia development (Moulton and Sollod 1976; Omotainse and Anosa 2009), whereby in particular the latter is one of the most prominent immunopathogenic features of both bovine and murine African trypanosomiasis. In this context, hosts displaying severe disease symptoms and pathogenicity are termed trypanosusceptible or susceptible, while hosts exhibiting an infection with low pathogenicity are referred to as trypanotolerant or resistant (Taylor 1998). This difference in susceptibility/resistance depends on both the parasite species/strain and the host (Naessens 2006). For the *T. brucei* infections it was shown that the C3H/HeN and BALB/c mice are more trypanosusceptible compared to the CBA/ca and C57Black/6 mice. In contrast, for the *T. congolense* infection BALB/c and CBA/ca mice were found to be susceptible while C3H/HeN and C57Black/6 mice are more trypanotolerant (Antoine-Moussiaux et al. 2008; Magez et al. 2004; Singh et al. 2013). Overall, studies into the role of cells of the mononuclear phagocyte system (MPS) which include myeloid cells (i.e., macrophages, monocytes and granulocytes) during experimental murine trypanosome infections (focussing on *T. brucei* and *T. congolense*) have revealed that these cells make an important contribution to African trypanosomiasis development both during the early and later/chronic stages of infection, whereby they can play a protective or pathogenic role depending on their activation state (Noel et al. 2004) (see Fig. 16.1). In particular, trypanotolerance is associated with induction of Type-1 cytokine responses [interferon-γ (IFNγ)] during the early phase of infection, giving rise to classically activated macrophages (M1) producing tumor necrosis factor (TNF) and/or nitric oxide (NO) and hereby ensuring parasite control, followed by switching to Type-2 cytokine production, including IL-4, IL-13, IL-10, during later phases of infection, giving rise to alternatively activated macrophages (M2) (Kaushik et al. 1999a; Stijlemans et al. 2007a). On the other hand, a maintained Type-1 cytokine response or an early mixed Type-1/2 cytokine production confers susceptibility to trypanosome infections (Stijlemans et al. 2007a; Baetselier et al. 2001; Namangala et al. 2001a). The ability of more resistant mouse strains to alleviate excessive infection-elicited pathogenicity thereby relies on a control of the Type-1-mediated immune responses, allowing a transition from parasite control towards pathogenicity control.

In the next sections we will discuss (1) the parasite–host interactions with a focus on the role played by cells of the MPS and parasite-derived components triggering immune responses during the different stages/phases of experimental trypanosome infections and (2) the contribution of cells of the MPS to immunopathogenicity development with focus on liver injury and anemia. Finally, we will give (3) an overview of different strategies that can be employed to alleviate immunopathogenicity which might pave the way to develop new intervention strategies, as well as (4) discuss the potential link between murine models and HAT.

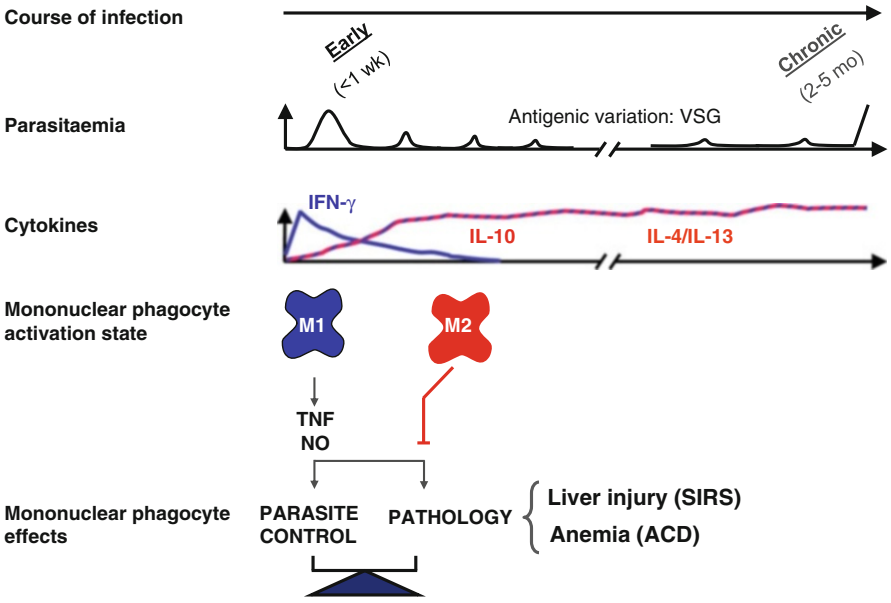

Fig. 16.1 Parasite control versus immunopathogenicity during African trypanosome infections. During the early stages of trypanosome infection the first wave of parasitemia is associated with the production of IFN-γ required for priming of mononuclear phagocytes. These activated mononuclear phagocytes (M1 cells) develop a Type-1 inflammatory immune response comprising the trypanocidal molecules TNF and NO, which in conjunction with antibodies contribute to parasite control. Yet, antigenic variation of the parasites VSG coat allows parasites to persist and to elicit new parasitic waves. In trypanosusceptible animals, a persistence of the Type-1 immune response and hyperactivated mononuclear phagocytes will culminate into immunopathogenicity features such as the systemic immune response syndrome (SIRS) and anemia of chronic disease (ACD). Yet, in trypanotolerant animals the host can mount a Type-2 immune response comprising sequential induction of IL-10 and IL-4/IL-13 which allow emergence of M2 cells with anti-inflammatory properties and hereby delay the onset of immunopathogenicity

16.2 Host–Parasite Interactions During the Course of African Trypanosomiasis

In order to better understand the role played by different trypanosome-released triggers and by mononuclear phagocytes in parasite development and in host–parasite interactions, it is important to delineate the different stages occurring during the course of African trypanosomiasis using C57Black/6 mice as model (see Fig. 16.2a).

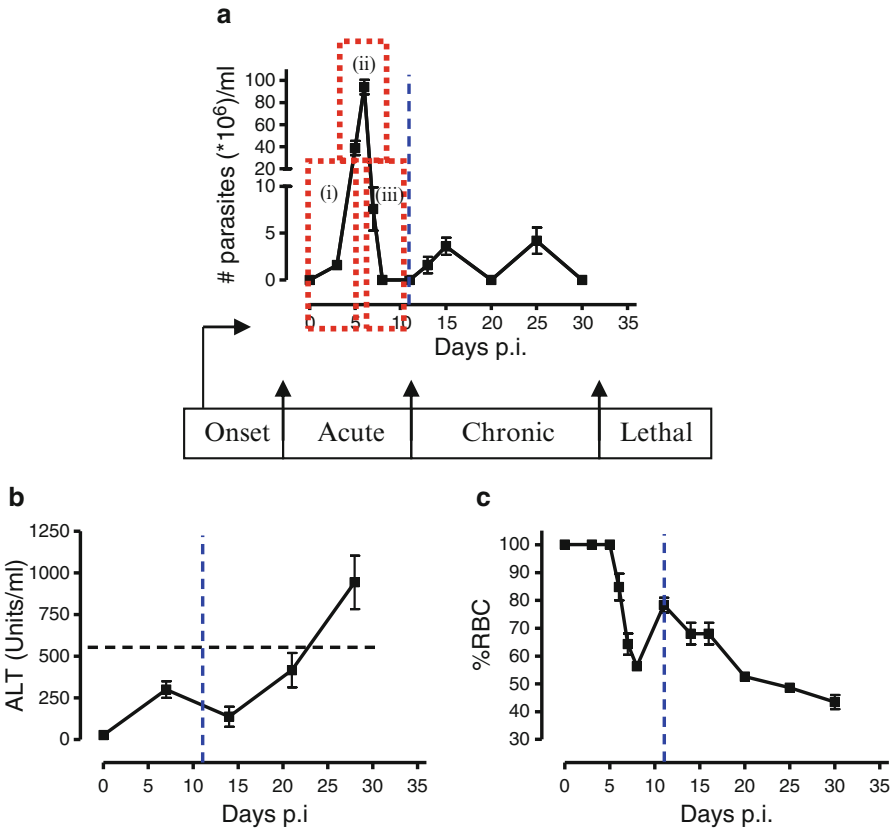

Fig. 16.2 Schematic overview of the different stages and phases of Trypanosoma brucei infections. (**a**) Parasitemia profile characterized by an onset stage (i.e., inoculation of parasites within the host), an acute stage, a chronic stage, and a lethal stage. The acute stage can be further subdivided into distinct phases whereby different parasite-derived components can trigger innate immune responses within the host; (i) ascending, (ii) peak, and (iii) descending phase. (**b**) Representative profile of liver pathogenicity measured by serum ALT levels during the course of trypanosome infections. (**c**) Representative anemia profile during the course of infection which is divided into an acute phase of anemia followed by a rapid recovery and a chronic/progressive phase

16.2.1 Subdivision of Experimental Trypanosome Infection into Distinct Stages/Phases

Stage 1 consists of the inoculation of *T. brucei* parasites into the host via an infected tsetse fly bite (natural mode of transmission) or via intra-peritoneal or intra-dermal injection of a parasite stabilate. Though at this onset stage *T. brucei*-derived components were so far not reported to play a direct role in promoting the infection, it is appropriate to mention that tsetse fly salivary components accelerate the

parasitemia onset by inhibiting local and systemic Type-1 inflammatory responses involved in parasite control (Caljon et al. 2006). Hence, already from the invasive step, protective innate defenses that rely on Type-1 molecules (IFN-α, TNF-α, and NO) and pro-inflammatory mononuclear cells (i.e., M1 cells) as well as on natural B cells are undermined (Lopez et al. 2008). Of note, inoculated trypanosomes via saliva of tsetse flies express a heterogenic metacyclic VSG coat which once into the mammalian host is changed into the first bloodstream form VSG (Modespacher et al. 1991). This is in contrast to parasite stabilate inoculation whereby all parasites express the same VSG.

Stage 2 or acute infection is characterized by the development of the first (most prominent) peak of parasitemia and its control by the host immune response. This stage can be subdivided into distinct phases, namely:

1. *The ascending phase* of parasitemia leading to the establishment of the parasites in the host, whereby the proliferating parasites face innate defense mechanisms of the host (mainly M1-derived TNF and natural IgM antibody) (Lucas et al. 1994; Magez et al. 1997) and require nutrient supply from the host (e.g., glucose, polyamines) (Nishimura et al. 2006; Milne et al. 1998). Overcoming these barriers leads to the first peak of parasitemia.
2. *The peak phase* of parasitemia implies extensive parasite control and destruction via the development of adaptive cellular and antibody responses during the transition from phase (1) to phase (2). In particular, antibody-mediated control of the first wave of parasites switches on in parasites the mechanism of antigenic variation and activation of membrane-bound phospholipase-C (PLC), leading to the release of VSG (soluble VSG/sVSG) (Carrington et al. 1991; Fox et al. 1986; Magez et al. 2008; Webb et al. 1997).
3. During *the descending phase* of parasitemia massive destruction of most of the trypanosomes occurs, culminating into the selection of parasites expressing another VSG and the release of extracellular and membrane-bound immune modulatory trypanosome components including, membrane-bound VSG (mfVSG) (Magez et al. 1998), its glycosyl-phosphatidyl-inositol (GPI) anchor (Magez et al. 1998, 2002; Stijlemans et al. 2007b) and the Trypanosome Suppression Inducing Factor (TSIF) (Gomez-Rodriguez et al. 2009) as well as CpG DNA (Mansfield and Paulnock 2005; Drennan et al. 2005).

At the peak and descending phase of parasitemia, some parasites differentiate into short-stumpy forms that are non-dividing forms pre-adapted for transmission to tsetse flies. But the majority is eliminated via the combined recognition of parasite-released factors, the differentiation and isotype switch of B cells and the production of the host cytokine IFN-γ, fuelling M1 cell activation exerting trypanotoxic activity (mainly TNF-α and NO mediated). Yet, as M1 cells become hyperactivated, collateral damage ensues (anemia, hepatic inflammation, increased blood–brain-barrier (BBB) permeability) (Stijlemans et al. 2010a; Bosschaerts et al. 2011; Untucht et al. 2011; Courtioux et al. 2006). Consequently, the final phase of stage 2 of infection results in the settlement of an antigenic *T. brucei* variant in certain anatomical niches such as the liver and the initiation of immunopathogenicity and immunosuppression.

Stage 3 or chronic stage of infection consists of sequential small peaks of parasitemia whereby, due to the mechanism of antigenic variation, *T. brucei* parasites change their VSG coat and elicit protective anti-VSG IgG antibodies. At this stage mixed type 1/2 cytokines and M1/M2 cells are elicited. At this stage external interventions aimed at promoting a clear M1–M2 switch seem to be beneficial for the host as evidenced by a prolonged survival and reduced pathogenicity (Stijlemans et al. 2007b; Guilliams et al. 2008, 2009) (see below).

Stage 4 is the lethal stage of infection. The reason(s) underlying lethality in experimental murine models are so far poorly known, even if evidence for SIRS initiated by hyperactivated cells of the MPS causing multiple organ failure accumulates (Shi et al. 2003).

16.2.2 Immunomodulatory Activities of T. brucei *Components During the Different Stages/Phases of an Experimental Infection*

Trypanosomes are equipped with a range of components that modulate the host immune response and which exert a dual effect: (1) they prevent their elimination in order to establish infection and as such allow parasite transmission or (2) they exert activating or inactivating effects on cells of the MPS of the host depending on the stage of the infection and thereby influence parasite control and/or pathogenicity development. In the following section, a number of examples of such factors, focussing on *T. brucei* components, are given. To date, *T. congolense* immunomodulators are poorly recognized besides VSG and a homologue of the major surface protease, gp63 of *Leishmania major* called Tco-MSP-D (Marcoux et al. 2010).

– *TbAdC*: *T. brucei A*denylate *C*yclase is an enzyme exposed on the trypanosome cell surface that converts ATP into cyclic adenosine monophosphate (cAMP) and pyrophosphate. The cAMP production in trypanosomes is known to be extremely low under basal conditions, however during stress reactions such as after phagocytosis by liver-associated M1 cells, TbAdC production is elevated to ~250 times the basal cellular content per minute (Rolin et al. 1996; Salmon et al. 2012). Consequently, host cAMP is produced in the phagocyte cytoplasm, activating a signal protein (i.e., protein kinase A) that inhibits production of the trypanocidal molecule TNF-α (Lucas et al. 1994; Magez et al. 1997). As a result these phagocytosed altruistic trypanosomes disable the M1-mediated innate immune response required to control parasitemia, clearing the way during the ascending phase of acute infection (stage 2/phase 1, Fig. 16.2a) for the parasites that follow.
– *CpG DNA*: In the acute stage of infection, low amounts of parasites are eliminated/lyzed during the ascending phase of parasitemia (stage 2/phase 1, Fig. 16.2a), releasing nuclear material into circulation and thereby triggering TLR9-signaling events that contribute to induction of M1 activation and the resulting inflammatory response needed for optimal first peak parasite control (Drennan et al. 2005;

Harris et al. 2006). The parasite DNA may also be involved in triggering B cells to proliferate and differentiate (Shoda et al. 2001; Shirota et al. 2002), thereby contributing to the differentiation of Th1 cells and isotype switching. Therefore, TLR triggering can be a critical step in the initiation of innate immunity against *T. brucei*.

– *sVSG, mfVSG, GPI*: During acute infection, both sVSG released via parasite PLC activity at the peak phase of parasitemia and mfVSG released during the descending phase of parasitemia (stage 2/phase 2 and 3, Fig. 16.2a) exert a TNF-α-inducing potential, provided cells of the MPS are primed by the host type-1 cytokine IFN-γ. Hereby, the GPI anchor of mfVSG and its moieties [glycosyl-inositol-phosphate (GIP) and dimyristoylglycerol (DMG)] play distinct, yet convergent roles (Magez et al. 1998, 2002). Briefly, the galactose residues of the sVSG–GIP (generated upon cleavage of mfVSG via parasite-derived PLC and leaving the DMG into the parasites' membrane) lead to activation of the protein tyrosine kinase (PTK) pathway and subsequent TNF production by IFNγ-activated mononuclear phagocytes (i.e., M1) (Tachado et al. 1997). In turn, the DMG compound of mfVSG-GPI (released during the descending phase of acute infection (stage 2/phase 3, Fig. 16.2a) and during the chronic stage of infection, see Fig. 16.1) activates the protein kinase C (PKC) pathway (Tachado et al. 1999). Collectively, these fuel the hyperactivation of M1 cells (TNF and IL-1 secretion) and LPS-hypersensitivity in a MyD88-dependent manner (Magez et al. 1998, 2002; Drennan et al. 2005), contributing to parasite control and M1-mediated collateral tissue damage. Interestingly, the involvement of the DMG compound in M1 over-activation has also been reported in experimental bovine models (Sileghem et al. 2001). It has been proposed that uptake of the sVSG molecule occurs in a specific, receptor-mediated fashion thereby triggering a signal transduction cascade, leading to NF-κB activation and the associated M1 activation needed for parasite control (Leppert et al. 2007).
– *TbTSIF*: *T. brucei T*rypanosome *S*uppressive *I*mmunomodulating *F*actor was identified as a protein probably released during the descending phase of the acute infection (stage 2/phase 3, Fig. 16.2a), that triggers the development of suppressive M1 cells which produce TNF and NO (Gomez-Rodriguez et al. 2009). TbTSIF-induced M1 block T-cell proliferation via a cell contact and IFN-γ/NO-dependent mechanism and down-regulate M2-oriented immune responses. Hence, TbTSIF represents a parasite molecule with the potential to modulate the host immune network, whereby it could contribute to the inflammatory response required to control parasite growth (TNF and NO) and to the pathogenicity of African trypanosomiasis, including immunosuppression. A thorough study of TbTSIF function in the outcome of infection could not be performed due to the fact that TbTSIF knock-out parasites were not viable and TbTSIF knock-down parasites died within 2 days. This indicates that TbTSIF is essential for *T. brucei* development/biology.

Collectively, there is evidence that trypanosomes are equipped with various tools such as TbAdC and TbTSIF to trigger signals by the host immune response in order to establish an infection and allow persistence within the host to sustain its lifecycle

(Paulnock et al. 2010). On the other hand, parasite-derived factors such as CpG DNA and VSG–GPI can provide strong pro-inflammatory M1-activating signals in conjunction with host factors such as IFNγ, leading to parasite control which in turn will fuel the highly polarized Th1 response to parasite antigens that develops during early infection (Paulnock et al. 2010).

16.3 Involvement of the MPS in Parasite Control and Pathogenicity Development

16.3.1 Involvement of M1 Cells in Parasite Control

To date, the main mechanism believed to mediate African trypanosome parasitemia control in the mammalian host is the continuous interplay between the parasites' surface, covered by about 10^7 identical densely packet VSG molecules, and the humoral immune response (i.e., antibodies directed against this VSG) (Pays 2006). Hereby, the clearing capacity of Abs during the early parasitemia control is most likely due to Ab-mediated phagocytosis of opsonized parasites by cells from the MPS, and has been amply documented in murine models as well as bovine infections for both *T. brucei* and *T. congolense* infections (Dempsey and Mansfield 1983; Shi et al. 2004; Morrison et al. 1982; Williams et al. 1996; Muller et al. 1996). Indeed, experimental murine in vivo models using B-cell (μMT) and IgM-deficient ($IgM^{-/-}$) mice revealed that during both *T. brucei* and *T. congolense* infections IgGs are crucial for parasite control, while IgM are dispensable for this process (Magez et al. 2006, 2008). These results are in accordance with livestock observations documenting the correlation between trypanotolerance and increased parasite-specific IgG production, while IgM antibodies were found to be mainly cross-reactive and the results of polyclonal B-cell activation (D'Ieteren et al. 1998). Hereby, IgG anti-VSG-mediated phagocytosis induced a stronger production of the trypanotoxic molecule NO by trypanosome-pulsed mononuclear phagocytes than IgM anti-VSG-mediated phagocytosis (Kaushik et al. 1999b, 2000). Of note, in murine models, >80 % of these extracellular opsonized parasites are eliminated by cells from the MPS within the liver. Besides Abs, there is compelling evidence that also other effector molecules are required for early parasite control. Hereby, IFN-γ produced by activated T-cells during *T. congolense* and *T. brucei* infections via an IL12p70-dependent but IL12p80 homodimer or IL23-independent mechanism was shown to be crucial in early parasite control due to its capacity to give rise to the development of M1 cells, which in turn produce trypanostatic or trypanotoxic components such as TNF, reactive oxygen species (ROS), and NO (Kaushik et al. 2000; Barkhuizen et al. 2007, 2008; Hertz and Mansfield 1999; Magez et al. 1999; Namangala et al. 2001b; Taiwo et al. 2002).

Evidence for the involvement of Type-1 effector molecules and M1 cells in parasite control was again provided using gene-deficient mouse models in C57Black/6

background (TNF$^{-/-}$, TNFR1 (p55)$^{-/-}$, TNFR2 (p75)$^{-/-}$, IFNγR$^{-/-}$, MHCII$^{-/-}$, and NOS2$^{-/-}$ mice). As far as *T. congolense* is concerned it was shown that (1) IFNγ-mediated M1 activation is crucial for parasite control, (2) signaling via MHC-II is required for IFNγ induction and subsequent TNF and NO production, (3) TNF and TNF-R1 (p55) signaling are required for first peak parasitemia control since mice deficient in either molecule cannot control the parasitemia and succumb early and (4) NO is crucial for peak parasitemia control (Magez et al. 2006; Lu et al. 2011). Therefore, in the *T. congolense* model it was suggested that IFNγ-mediated TNF induction followed by binding to TNF-R1 triggers iNOS activation resulting in the production of trypanostatic NO (Magez et al. 2007). This in turn would reduce the "in vivo" fitness of the parasites which in turn would facilitate the IgG Ab opsonization and subsequent mononuclear phagocytes mediated phagocytosis leading to efficient clearance (Shi et al. 2004; Magez et al. 2006). In the *T. brucei* model, IFNγ was also shown essential for resistance to infection, whereby IFNγ$^{-/-}$ mice exhibit higher parasitemia levels and die earlier than WT mice (Namangala et al. 2001b; Hertz et al. 1998). Yet, the documented "in vitro" trypanotoxic effect of NO on *T. brucei* does not seem to affect the in vivo parameters such as parasitemia control or survival (Hertz and Mansfield 1999; Vincendeau et al. 1992). In this model, another molecule seems to affect parasite fitness, namely TNF. Indeed, this molecule was shown to exhibit a direct trypanolytic effect on *T. brucei* upon binding to VSG at least in vitro (Magez et al. 1997; Daulouede et al. 2001). This is in contrast to *T. congolense* parasites which are completely refractory to TNF (Magez et al. 2006, 2007). Also in vivo TNF was found to be crucial for *T. brucei* parasite control, whereby TNF$^{-/-}$ mice exhibit significantly higher parasite numbers as compared to WT control mice (Magez et al. 1999).

Collectively, efficient *T. brucei* or *T. congolense* parasite clearance from the circulation involves three different effector mechanisms; (1) an IFNγ-driven M1 activation of cells from the MPS resulting in the induction of trypanotoxic compounds (TNF or NO) needed for reducing the in vivo parasite fitness, (2) recognition of parasites by VSG-specific antibodies (IgG and IgM), and (3) opsonization and subsequent phagocytosis of damaged and antibody-covered parasites by cells from the MPS. The liver-associated mononuclear phagocytes have long been recognized as the first host immune cells to encounter trypanosomes and/or their products during natural as well as experimental infections, thereby orchestrating the onset of infection (Askonas 1985; Grosskinsky and Askonas 1981; Grosskinsky et al. 1983).

16.3.2 Involvement of M2 Cells in Limiting Tissue Pathogenicity

The liver is a highly specialized tissue playing a key role in detoxification, protein (plasma protein and hormone) synthesis, glucose, and lipid homeostasis as well as in removal of senescent RBC and pathogens. In the context of African trypanosome infections, it is generally accepted that upon phagocytosis of trypanosomes by cells

from the MPS within the liver and depending on the degree of cell activation, there is occurrence of hepatomegaly, focal liver lesions and apoptosis of parenchymal cells, enlargement of the capillary bed and hypotension (Shi et al. 2003, 2005, 2006). The excessive/persistent activation of M1 and IFNγ-producing T-cells observed in trypanosusceptible animals leads to liver damage (illustrated in Fig. 16.2b) and shortens the survival of infected hosts (Magez et al. 2004; Shi et al. 2003, 2004). Hereby, the crucial role of TNF in the occurrence of immunopathogenicity and the protective role of IL-10 (i.e., keeping the balance between pathogenic and protective immune responses) have been extensively studied. In the *T. brucei* model, which is locked in a Type-1 immune response (M1 cells) and cannot sustain IL-10, $TNF^{-/-}$ mice exhibited reduced liver damage. In contrast, using $IL\text{-}10^{-/-}$ mice it was shown that IL-10 plays a key role in survival (8 days p.i. in $IL\text{-}10^{-/-}$ mice versus 5 weeks in WT mice) and in controlling excessive inflammatory responses without influencing parasite load control mechanisms (Guilliams et al. 2009; Namangala et al. 2001b). Furthermore, a phospholipase-C deleted *T. brucei* strain (*T. brucei PLC*$^{-/-}$) gives rise to a chronic infection, whereby an early IFN-γ-dependent M1 activation followed by a gradual switch towards an IL-10-mediated M2 activation coincides with an alleviated immunopathogenicity and a significantly prolonged survival (up to 5–6 months) (Baetselier et al. 2001; Namangala et al. 2000, 2001a). In the *T. congolense* model it was shown that (1) treatment of infected C57Black/6 mice with anti-IL10R blocking antibodies or depletion of IL-10 producing naturally occurring $Foxp3^{+}$ Tregs abrogated the IL-10 suppression on M1 activation of the CD11b(+) Ly6C(+) monocytic cell subsets of the MPS at the level of TNF and NO production and the trypanotolerance, resulting in increased liver injury and shortened survival (Guilliams et al. 2009). Moreover, absence of IL-10 producing myeloid cells also contributes to the development of a trypanosusceptible phenotype (Bosschaerts et al. 2011; Guilliams et al. 2007).

Collectively, these data indicate that during African trypanosomiasis a switch from M1 towards M2 during the course of infection has to be timely regulated, whereby the early IFN-γ-mediated M1 activation needed for initial peak parasitemia control is followed by switching towards an IL-10-driven induction of M2 during the chronic stage of infection (Noel et al. 2002). Important to mention is that although both IL-4 and IL-13 are also known to induce M2 activation of cells of the MPS (Gordon and Martinez 2010) and are produced during the chronic stage of *T. congolense* and *T. brucei PLC*$^{-/-}$ infection (see Fig. 16.1), using $IL4^{-/-}$ and $IL4Ra^{-/-}$ mice it was shown that they do not influence the pathogenicity in the trypanosome models studied so far (Namangala et al. 2000; Noel et al. 2002; Schopf et al. 1998). It should also be remarked that, in addition to limiting local tissue damage caused by exaggerated inflammation and resulting in a prolonged survival, IL-10 produced by Treg cells or cells of the MPS can also prevent cure via inhibition of antigen-specific immune responses and thus favor parasite persistence (Trinchieri 2007). In fact, both M1 and M2 can exert suppressive activities, but their suppressive activities differ. For instance, M1 can inhibit T-cell activation triggered by trypanosome-unrelated antigens, while M2 impair only antigen-specific induced T-cell proliferation (Baetselier et al. 2001; Namangala et al. 2001a). Hereby, the suppressive activity of M1 mainly

depends on prostaglandin (PG) and NO synthesis (Schleifer and Mansfield 1993). In contrast, the suppressive activity of M2 may involve IL-10 (Uzonna et al. 1998).

Given the importance of M2 in preventing immunopathogenicity development and hence their association with trypanotolerance in the African trypanosome model we have scrutinized this model to characterize the molecular repertoire and mechanisms underlying the M2 functional properties. By performing a comparative gene expression analysis between the in vivo elicited M2 with M1 in the attenuated *T. brucei* PLC$^{-/-}$ model and subsequently comparing these genes with various M2 populations elicited in murine models of infectious diseases (i.e., *T. congolense*, helminths) as well as cancer, a common gene-signature for in vivo elicited M2 could be obtained independent of the disease model, the mouse strain, and organ source of the cells (Noel et al. 2004; Ghassabeh et al. 2006). Hereby, some of these genes were not induced by IL-4/IL-13 or IL-10 under various "in vitro" settings, illustrating that "in vivo" conditions can generate alternative M2 triggers. However, considering the essential role of IL-10 in trypanotolerance (see above), M2-related genes that are IL-10 inducible and expressed differentially between trypanosusceptible and trypanotolerant models of *T. congolense* infection were further investigated. This allowed the identification of eight genes that were either IL-10 inducible in vitro in cells of the MPS from non-infected animals (*Arg1*, *Sepp1*, *Mgl1*, *Mrc1,* and *Folr2*) (Ghassabeh et al. 2006) or IL-10 dependent during *T. congolense* infections (*Sepp1*, *Ctss1*, *F12a1, and Ngfb*) (Bosschaerts et al. 2008). One of these candidate genes, *sepp1* (which encodes selenoprotein P, i.e. a protein exhibiting anti-oxidant properties), was shown to limit tissue injury by limiting production of ROS and mononuclear phagocyte apoptosis in the liver, preserving their parasite clearance capacity (Bosschaerts et al. 2008). The protective role of selenoprotein P also relied on its ability to limit ROS production, therefore preserving liver parenchymal cells from type-1 inflammation-mediated apoptotic/necrotic damage. Therefore, the identification of these M2-associated genes and their products may represent targets for therapeutic interventions and may be of generic value for infectious diseases where immunopathogenicity plays a crucial role. Yet, a better understanding/identification of the role of M1 cells during the different stages of infection could also pave the way to reduce immunopathogenicity.

16.3.3 Involvement of Liver Monocytic Cells in Pathogenicity

Within mononuclear phagocytes, two distinct subsets of murine monocytic cells have been distinguished: inflammatory and patrolling monocytes characterized by their CD11b^{+}Ly6C^{high}CX3CR1^{low}CCR2^{high}CD62L^{+} and CD11b^{+}Ly6C^{low}CX3CR1^{hi}CCR2^{-}CD62L^{-} expression, respectively (Saha and Geissmann 2011). These monocytes migrate from the bone marrow to the blood circulation and subsequently to inflamed organs following inflammation and complement the resident mononuclear

phagocyte/macrophage pool (Serbina et al. 2008). During African trypanosome infection, we observed that inflammatory CD11b^{+}Ly6C^{high} monocytes gradually accumulate into the liver (also in spleen and lymph nodes). These monocytes were found to express besides CCR2, different chemokine receptors such as CCR5 and CD74 (i.e., the receptor of MIF, Macrophage Migration Inhibitory Factor). A proportion of these CD11b^{+}Ly6C^{high} cells was found to differentiate into CD11b^{+}Ly6C^{+}CD11c^{+} inflammatory DCs which express CD80/86, MHC class II (i.e., indicative for a mature phenotype) and form a major source of TNF and iNOS during *T. brucei* as well as *T. congolense* infections (Bosschaerts et al. 2010, 2011; Guilliams et al. 2009), which classifies them as Tip-DCs (Serbina et al. 2003). In addition, it was found that the conversion of CD11b^{+}Ly6C^{high} monocytic cells to Tip-DCs in the liver of *T. brucei* infected mice consisted of a three-step process including (1) a CCR2-dependent but CCR5- and MIF-independent step crucial for emigration of CD11b^{+}Ly6C^{high} monocytic cells from the bone marrow but dispensable for their blood to liver migration; (2) a differentiation step of liver CD11b^{+}Ly6C^{high} monocytic cells to immature inflammatory DCs (CD11c^{+} but CD80/CD86/MHC-II^{low}) which is IFN-γ and MyD88 signaling independent; and (3) a maturation step of inflammatory DCs to functional (CD80/CD86/MHC-II^{high}) TNF and NO producing Tip-DCs which is IFN-γ and MyD88 signaling dependent (Bosschaerts et al. 2010).

A pro-pathogenic function for recruited CD11b^{+}Ly6C^{high} monocytic cells/Tip-DCs is supported by the observation that in CCR2 KO mice or in *T. brucei* infected mice treated with IL-10 (Guilliams et al. 2009), a reduced percentage of recruited CD11b^{+}Ly6C^{high} monocytic cells/Tip-DCs was associated with reduced pathogenicity and increased survival time without affecting parasite control. In addition, in mice lacking IL-10R signaling, TNF/iNOS-producing CD11b^{+}Ly6C^{high} cells increased in the liver and were associated with increased liver destruction and reduced survival time (Bosschaerts et al. 2010, 2011). Furthermore, IL-10 could inhibit the CCL2/CCR2-mediated egression of CD11b^{+}Ly6C^{high} monocytic cells from the bone marrow by limiting *Ccl2* expression by liver monocytic cells as well as their differentiation and maturation to Tip-DCs in the liver (Bosschaerts et al. 2010). Hence, liver injury can result from uncontrolled activation of monocyte-derived cells recruited to the liver via CCR2 signaling and can be modulated by IL-10 (Karlmark et al. 2009). In particular, in *T. congolense* infected mice, IL-10 was found to favor nuclear accumulation of the NF-κB p50 subunit over the p65 subunit in monocyte-derived cells and hereby to limit the production of pathogenic TNF in an autocrine loop (Bosschaerts et al. 2011). The role of the other monocyte subset CD11b^{+}Ly6C^{low} monocytes in the outcome of trypanosome infection is currently under investigation.

Based on the above results obtained from murine models of trypano-tolerance and -susceptibility to African trypanosomiasis, i.e. based on *T. brucei* and *T. congolense*, a tentative model accounting for the resistance to African trypanosomiasis in mice can be proposed (see Fig. 16.3).

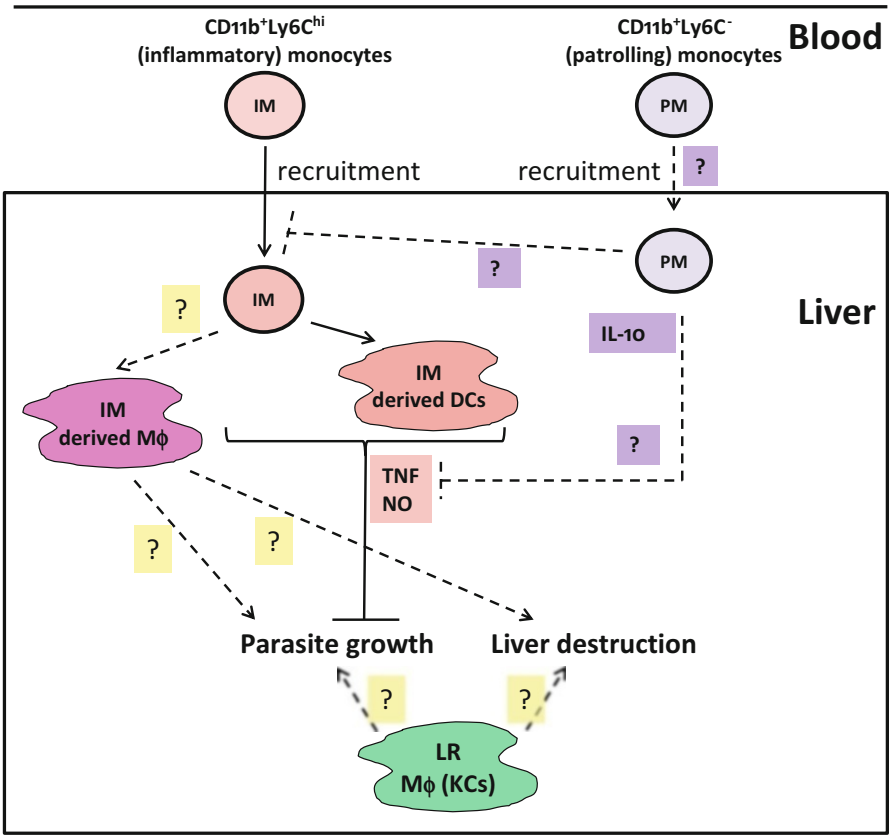

Fig. 16.3 Role of tissue-associated mononuclear phagocytes in resistance and tolerance to African trypanosome infection: a working model. $CD11b^{+}Ly6C^{hi}$ monocytes exiting the bone marrow in a CCR2/CCL2-dependent pathway are recruited into the liver of infected mice and differentiate into TIP-DCs. These cells exhibit an M1 activation status, producing the trypanotoxic compounds TNF and NO that contribute to the resistance to infection (control of parasite growth). Simultaneously, these cells exert pathogenic activity and decrease the tolerance to infection (cause tissue damage). IL-10 produced by cells of the mononuclear phagocyte system (MPS) is essential to restrict the pathogenic activity of M1-oriented cells. In this context, a number of questions remain unanswered. For instance, what is the role of the other major monocyte subset, the $CD11b^{+}LyC6^{-}$ monocytes, in the outcome of infection? Do recruited $CD11b^{+}Ly6C^{hi}$ monocytes differentiate into M1 or M2-type macrophages and hereby affect the resistance and tolerance to infection? What is the role of liver resident (LR) macrophages (Kupffer cells, KCs) to parasite control and liver destruction?

16.3.4 Involvement of Mononuclear Phagocytes in Anemia Development

Another prominent pathogenic parameter during African trypanosomiasis is anemia development. In particular in domestic livestock populations anemia is considered to be the major cause of death and the capacity to limit anemia is a critical parameter in determining trypanotolerance. In cattle, anemia development was found to be

independent of parasitemia control, T-cells, and antibodies (Naessens 2006; D'Ieteren et al. 1998; Murray et al. 1982, 1990; Murray and Dexter 1988). To unravel the mechanisms underlying trypanosomiasis-elicited anemia development, murine models, exhibiting different degrees of anemia development, were scrutinized.

As far as the *T. brucei* model is concerned, anemia development (illustrated in Fig. 16.2c) was found not to correlate with parasitemia control or survival time (Naessens 2006; Nakamura et al. 2003), similar as for bovine trypanosomiasis (Sileghem et al. 1994), suggesting that anemia development is a consequence of host immune responses rather than a direct influence of parasite products. Indeed, TNF, TNF-R2, IFNγR, and MyD88-deficient mice exhibited significantly lower anemia levels and higher parasitemia levels as compared to control wild-type C57Black/6 mice (Magez et al. 1999, 2004; Drennan et al. 2005). It was suggested that the MyD88-dependent activation of innate immune responses resulted in the induction of IFNγ and subsequent TNF induction, whereby TNF-R2 signaling plays a key role in the induction of pathogenicity. In fact, the increased ratio of TNF over its soluble receptor 2, not TNF levels per se, relates to the occurrence of infection-associated anemia (Magez et al. 2004). Thus, M1 hyperactivation was proposed to be the main mechanism involved in the extra-vascular destruction of red blood cells (RBCs) due to massive erythrophagocytosis in spleen and liver by activated mononuclear phagocytes of the trypanosome-infected host (Naessens 2006; Shi et al. 2004, 2005; Murray and Dexter 1988; Andrianarivo et al. 1996; Anosa and Kaneko 1983). In addition, anemia development was found to be B-cell independent (Magez et al. 2008).

Also in infected cattle, the observation of hyperactivated mononuclear phagocytes and erythrophagocytosis in tissues suggests that they may be major causes of anemia (Naessens et al. 2003; Taiwo and Anosa 2000; Anosa et al. 1997). In addition to a direct contribution of cells of the MPS to a reduction in RBCs via erythrophagocytosis, these cells can also contribute indirectly to anemia via modulation of erythropoiesis and iron homeostasis. In fact, the anemia induced during African trypanosomiasis in mice relates to the so-called anemia of chronic disease or ACD that is associated with chronic infections and inflammation [reviewed by Weiss et al. (Weiss 2009; Weiss and Goodnough 2005)], is characterized by an imbalance between erythrophagocytosis and erythropoiesis, and is linked to a perturbed iron homeostasis including altered iron recycling by macrophages and iron sequestration. Hereby, under physiological conditions tissue mononuclear phagocytes, in particular liver-associated mononuclear phagocytes (likely Kupffer cells and/or Ly6c$^+$ monocytes and derived macrophages) recover ferrous iron (Fe^{2+}) via engulfment of senescent RBCs from the circulation. In addition, hemoglobin is internalized and iron (Fe^{2+}) is extracted through the action of heme-oxygenase-1 (HO-1), resulting in the release of ferrous iron (Fe^{2+}), carbon monoxide, and biliverdin (Wunder and Potter 2003). Iron (Fe^{2+}) is then transported from the phagosome into the cytosol via the divalent metal transporter-1 (DMT-1 also called Nramp-2), the main Fe^{2+} transporter, from where it can be either stored intracellularly via ferritin (FHC) or exported extracellularly via ferroportin-1 (FPN-1) depending on the demand for iron of the host [reviewed in Weiss et al. (Weiss and Goodnough 2005)].

The extracellular Fe^{2+} will, upon conversion to ferric iron (Fe^{3+}) through ceruloplasmin (CP), be bound to transferrin (Tf) and transported, mainly to the bone marrow to fuel erythropoiesis. Consequently, limitation in ferric iron (Fe^{3+}) availability may exert a strong negative impact on erythropoiesis and contribute to ACD (Tilg et al. 2002). Our results suggest that during the course of *T. brucei* infection in mice, anemia development can be divided into two phases [see Fig. 16.2c and reviewed by Stijlemans et al. (Stijlemans et al. 2008, 2010b)]: (1) an acute/aggressive phase whereby M1-activated cells of the MPS play a key role in eliminating/phagocytosing RBCs mainly in the liver, resulting in an activation of pathways that govern iron-homeostasis and (2) a short partial recovery followed by a chronic/progressive phase whereby persistence of M1 cells reduces iron bio-availability to storage sites within the reticuloendothelial system, thereby diverting iron needed for erythropoiesis. In parallel, the enhanced uptake of RBC and iron-containing compounds is maintained, which in turn aggravates anemia development and prevents recovery to the normal RBC numbers (Stijlemans et al. 2008). Recently, we found that Galectin-3 (Gal-3), a lectin documented to contribute to the onset and persistence of type 1 inflammatory responses and phagocytosis (Sano et al. 2003) was strongly upregulated during *T. brucei* infection and involved in trypanosomiasis-associated anemia development (Stijlemans et al. 2008). Hereby, galectin-3 deficient ($Gal3^{-/-}$) mice exhibited greatly reduced inflammation-associated anemia development coinciding with a restored iron-homeostasis and increased IL-10 levels during *T. brucei* infection, that in turn may also lead to reduced liver malfunction and destruction.

As far as the *T. congolense* model is concerned, it seems that during the early stages of infection there is an increased uptake of erythrocytes for degradation, reduced uptake of haptoglobin, and decreased export of iron from the liver similar to the *T. brucei* model (Noyes et al. 2009). Here, insoluble hemosiderin rather than ferritin might restrict the availability of iron for erythropoiesis. However, further studies are required to determine whether iron stored as insoluble hemosiderin is restricting its availability for erythropoiesis (Stijlemans et al. 2008). During the later phases of *T. congolense* infection, anemia was found to be TNF-independent (Naessens et al. 2005). Hereby parasite components (sialidases/trans-sialidases) were documented to directly affect RBC and hence could contribute to anemia development (Coustou et al. 2012). Interestingly, also in *T. congolense* infected cattle evidence has been provided for the contribution of other mechanisms in anemia development, such as production of hemolysins by the parasites [reviewed in (Murray et al. 1982; Murray and Dexter 1988)], differences in type and amounts of sialic acids (Esievo et al. 1990; Nok and Balogun 2003), binding of autologous or polyreactive antibodies or complement C3 to erythrocyte surfaces (Kobayashi and Tizard 1976) or the passive absorption of trypanosome molecules in the erythrocyte membrane (Rifkin and Landsberger 1990). Therefore, anemia associated with trypanosome infections is multi-factorial and the relative contribution of each mechanism will differ according to the host–parasite model, the phase of anemia development, and the severity of infection.

Thus based on the murine models analyzed so far, two main types of anemia can be considered, namely host (inflammation)-mediated anemia and parasite-induced anemia (see proposed model, Fig. 16.4).

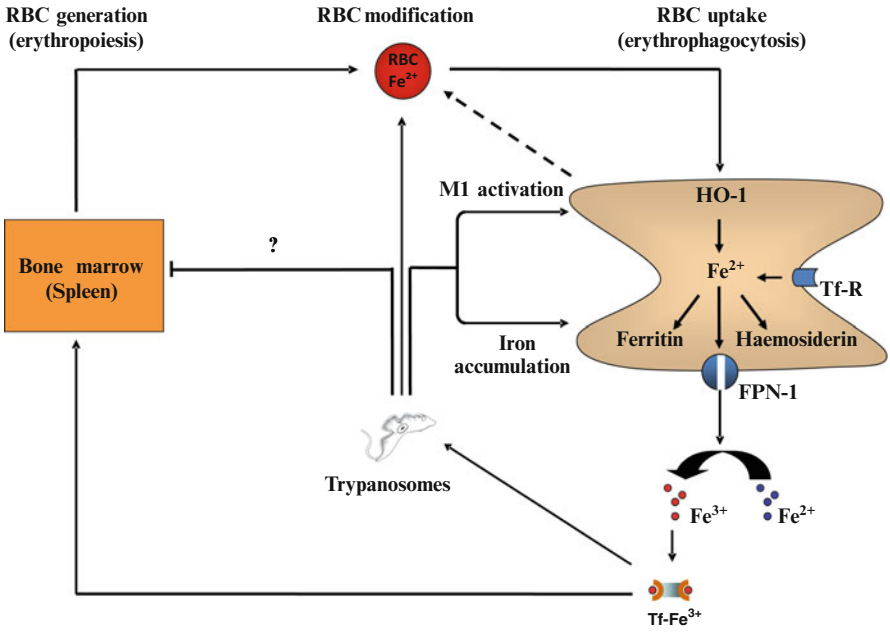

Fig. 16.4 Potential mechanism underlying trypanosomiasis-associated anemia. Upon infection cells of the MPS are activated giving rise to M1 cells and due to their higher phagocytic activity, erythrophagocytosis of damaged/senescent RBC will be increased, M1 released molecules such as TNF can reduce the half-life of RBC and thereby indirectly contribute to enhanced erythrophagocytosis, direct parasite-mediated RBC modification via parasite-derived components (sialidases/trans-sialidases or the passive absorption of trypanosome molecules in the erythrocyte membrane) may occur leading to a higher susceptibility for erythrophagocytosis. Following engulfment and catabolism of RBC by activated cells of the MPS ferrous iron (Fe^{2+}) will be extracted from hemoglobin through the action of heme-oxygenase-1 (HO-1). Subsequently, Fe^{2+} can be stored inside the cell via ferritin or hemosiderin or exported outside the cell via Ferroportin-1 (FPN-1) depending on the needs of the host. Exported Fe^{2+} is converted to Fe^{3+} and subsequently bound to transferrin (Tf) to be transported throughout the body where iron is needed, mainly the bone-marrow (spleen during inflammation) where it is used for erythropoiesis. During the course of trypanosome infections the Fe^{2+} is retained within the cells of the MPS in ferritin/hemosiderin resulting in the deprivation of iron needed for erythropoiesis. Also trypanosomes can acquire Tf-bound iron, thereby contributing indirectly to deprivation of iron from erythropoiesis. A direct/indirect negative influence of trypanosomes on erythropoiesis cannot be excluded

It should be remarked that, in addition to a role in anemia, iron may also play a key role with respect to the M1-mediated pathogenicity, since it is a well-known suspect in oxidative tissue damage (Wlaschek and Scharffetter-Kochanek 2005). Indeed, increased liver iron accumulation within mononuclear phagocytic cells can lead to or contribute to increase morbidity (Musallam et al. 2011; Xiong et al. 2004). In addition, it was shown that intracellular iron stimulates continuous mononuclear phagocyte activation by activating the major transcription factor NF-κB and primes the liver for chronic inflammation and injury (Xiong et al. 2003). Recently, it was even shown that iron overloading in mononuclear phagocytes is the major environmental cue responsible for the persistence of an unrestrained pro-inflammatory M1 activation state (Sindrilaru et al. 2011).

16.4 Strategies Aiming at Reducing African Trypanosomiasis-Associated Immunopathogenicity

The critical requirement of the switch from M1 during early stages of infection towards M2 during later stages and the pivotal role of IL-10 at preventing immunopathogenicity both at the level of liver injury and anemia development have been amply documented. Hereby, the observations from the *T. congolense* model in C57Black/6 mice whereby both M2-type cells and $CD4^+CD25^+Foxp3^+$ natural occurring Tregs can be considered as major sources of IL-10 formed the basis for potential intervention strategies aiming at inducing trypanotolerance in models associated with a persistent M1 activation state, absence of Tregs and M2 cell expansion and transient induction of IL-10. Several strategies were validated within the *T. brucei* model such as (1) stimulation of Treg expansion through a CD28 superagonist antibody treatment (Guilliams et al. 2008), (2) inducing sustained IL10 levels using an AAV (adeno-associated viral vector) gene delivery system (Guilliams et al. 2009), or (3) a GPI-based treatment strategy (i.e., macrophage reprogramming strategy) (Stijlemans et al. 2007b). With respect to the first two strategies, the protective effect resides in the fact that both treatments down-regulate the production of IFNγ (by $CD4^+$ and $CD8^+$ T-cells), reduce the differentiation and maturation of $CD11b^+Ly6c^{high}$ monocytes into TIP-DCs in the liver and other organs and induce M2-type cells, which in turn associate with (1) reduced liver injury, (2) reduced anemia, and (3) prolonged survival. This IL-10-mediated suppression of monocyte differentiation is reversible since monocytes from *T. brucei* infected IL-10 treated mice transferred to infected recipient mice in absence of IL-10 differentiate rapidly into TIP-DCs (Guilliams et al. 2009). This suggests that IL-10 is constantly required to control the long-term TIP-DC-mediated inflammation. The third strategy did not elicit the development of Tregs. However, its protective effect resides in its capacity to reprogram/desensitize macrophages by limiting the maturation step of inflammatory TIP-DCs (Stijlemans et al. unpublished) and giving rise to M2 cells (Stijlemans et al. 2007b). Moreover, the GPI-based treatment strategy was also found to alleviate anemia development in the *T. congolense* and *T. evansi* model systems as well as in tsetse fly challenge experiments, indicating potential field applicability for this intervention strategy. Furthermore, a comparative genetic analysis of mononuclear phagocytes between trypanosusceptible mice and trypanoresistant mice confirmed that trypanotolerance was associated with a restored expression of genes involved in iron-homeostasis and erythropoiesis (Stijlemans et al. 2010a). It is important to mention that, despite their capacity to reduce the TIP-DC maturation, neither of the treatments described above in the *T. brucei* model exacerbated parasite growth (Stijlemans et al. 2007b; Guilliams et al. 2009), suggesting that early stage M1 mononuclear phagocyte activation can be dampened to a certain extent without impairing control of parasite growth. Therefore, it seems that the major factor determining survival during African trypanosomiasis resides in the ability of the host to limit tissue injury. It should hereby be remarked that even trypanotolerant animals (i.e., exhibiting enhanced levels of M2 cells) although surviving longer finally die after several months post infection. The exact reason why these mice die remains elusive.

16.5 Perspectives

Up to now, most research focussed on unraveling the role of recruited CD11b^{+}Ly6c^{high} monocytic-derived cells during the course of trypanosome infection with respect to their role in parasite control and pathogenicity development. Yet, the role of the other CD11b^{+}Ly6c^{low} monocytic subset, of monocyte-derived macrophages, and of resident macrophages including Kupffer cells still remains unaddressed. Hereby, it cannot be excluded that recruited and resident mononuclear phagocytes in the liver respond/cooperate differently to the elimination of trypanosomes as far as phagocytic activity and production of trypanotoxic compounds such as TNF and NO is concerned. In addition, they might also contribute differently as far as RBC elimination is concerned. Furthermore, not only mononuclear phagocytes might contribute to the enhanced RBC clearance but also hepatic sinusoidal endothelial cells (HSECs) play a key role in effectively clearing damaged RBCs in mouse liver (Lee et al. 2011). Therefore, better characterization of each of these different myeloid subsets is required in order to obtain a complete picture of the potential players involved in immunopathogenicity development during African trypanosomiasis. Furthermore, the anemia occurring during trypanosome infections and more particular in the *T. brucei* model resembles ACD, which is the most important form of anemia occurring in hospitalized patients and develops under chronic inflammatory disorders such as infections, cancer, or autoimmune diseases (Weiss 2002). In addition, the impaired iron recirculation from mononuclear phagocytes may be central in the pathogenicity of ACD (Theurl et al. 2006, 2009). Hence, better understanding of the underlying mechanisms involved in anemia induced in experimental African trypanosomiasis might pave the way for a more general treatment of this form of anemia.

It is important to keep in mind that mice are not natural hosts of trypanosomes and therefore, more research needs to be performed before extrapolating the findings to human or bovine natural infections. Indeed, there are differences between murine, bovine, and human trypanotolerance (Naessens 2006; Kennedy 2007, 2013; Sternberg 2004). For example, the level of parasitemia is extremely high in mouse models as compared with those found in natural hosts. Nevertheless, during both natural and experimental African trypanosome infection, the host's immune response, rather than the parasite load throughout infection seems to be the major cause of peripheral tissue pathogenicity, resulting in the fatal outcome of the infection. Therefore, these mouse models have contributed significantly to our understanding of trypanosomiasis as a disease. Interestingly, there was a correlation between decreased pathogenicity of bovine strains and a decreased secretion of NO by IFNγ-activated peripheral blood mononuclear cell (PBMC) and an increased transcription of IL-10 (Taylor 1998). Furthermore, the capacity to control anemia and the erythropoietic potential are thought to be the most significant factors contributing to trypanotolerance of cattle (Andrianarivo et al. 1996; Naessens et al. 2005). In HAT, anemia has been observed, yet the major pathogenic feature is neuropathogenicity. During both *T. b. rhodesiense* and *T. b. gambiense* infection, there is an infiltration of parasites through the BBB due to the local production of

IFN-γ as well as TNF and NO (Grab and Kennedy 2008), thereby increasing the permeability of the endothelial cell layer of the BBB (Kennedy 2007). In addition, IL-10 in combination with IL-6 was also shown to be associated with reduced brain injury during African trypanosomiasis (Sternberg et al. 2005), suggesting that as in murine models also here IL-10 seems to have a regulatory function in dampening immunopathogenicity. As the disease progresses, HAT patients might develop various features including lymphadenopathy (enlargement of the spleen and liver), cardiac features (myocarditis, pericarditis, and congestive heart failure), ophthalmological features (iritis, keratitis, and conjunctivitis) as well as endocrine dysfunctions (impotence, menstrual abnormalities) (Brun et al. 2010; Blum et al. 2012; Carod-Artal 2010). Although the liver failure during HAT is usually moderate, other signs of liver problems have been reported, like jaundice hyper-bilirubinemia, and ascites (Blum et al. 2012; Kouchner et al. 1979). Moreover, studies focusing on liver immunopathogenicity induced by these parasitic infections may allow unraveling the underlying mechanisms and identifying new therapeutic leads to treat parasite-induced injury in particular and hepatic inflammation in general.

Recently, a functional genomics approach was evaluated to identify temporal changes in cattle PBMC gene expression due to disease progression. This approach revealed that major gene expression differences exist between cattle from trypanotolerant and trypanosusceptible breeds (Hill et al. 2005; O'Gorman et al. 2009). This type of approach might pave the way to identify crucial genes/pathways associated with trypanosusceptibility/trypanotolerance and allow more efficient cross-breeding schemes.

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Chapter 17
Macrophages in Obesity and Insulin Resistance

G. Chinetti-Gbaguidi and B. Staels

17.1 Introduction

Obesity is a chronic low-grade inflammatory disease (Vandanmagsar et al. 2011). Circulating monocytes infiltrate adipose tissue during the development of obesity where they differentiate into adipose tissue macrophages (ATM) (Weisberg et al. 2003). ATM, together with other non-adipocyte cell types in adipose tissue (pre-adipocytes, endothelial cells, lymphocytes, dendritic cells), are found in the stromal vascular fraction (SVF). Flow cytometry analysis revealed that macrophages constitute approximately 10 % of SVF cells in lean mice, reaching 40 % in obese animals (Weisberg et al. 2003). Similarly, in humans, the number of macrophages in the SVF increases proportionally to the body mass index (BMI) (Curat et al. 2006).

To understand the role of ATM in obesity and insulin resistance, it is important to distinguish between the different adipose depots, such as visceral and subcutaneous adipose tissue. More macrophages accumulate in visceral adipose tissue, which surrounds internal organs, than in subcutaneous fat depots. Additionally, while the total number of macrophages increases during obesity, this increase is more important in the visceral adipose tissue compartment (Cancello et al. 2006; Altintas et al. 2011). Visceral ATM accumulation is associated with hepatic inflammatory lesions in human morbidly obese subjects, while the accumulation of subcutaneous fat mass seems not to influence the hepatic phenotype (Cancello et al. 2006).

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Macrophages are not uniformly localized within the visceral adipose tissue. They form particular structures surrounding death adipocytes, called "crown-like structures" (CLS) (Cinti et al. 2005). The number of CLS increases in visceral adipose tissue of high fat-diet fed mice (Murano et al. 2008). In obese humans, the number of CLS is greater in visceral than in subcutaneous adipose tissue and it correlates with local inflammation and insulin resistance (Apovian et al. 2008). In addition, the lipid content of ATM is higher in visceral adipose tissue, compared to subcutaneous fat, demonstrating that macrophage foam cells, classically studied in vascular atherosclerosis, are also present in human adipose tissue, particularly in omental fat (Shapiro et al. 2013). Very recently, using an original siRNA delivery technology developed to silence gene expression specifically in ATM of obese mice (without modifying macrophages in other tissues, such as liver, heart and pancreas), knockdown of the inflammatory cytokines TNFα and osteopontin 1 improved insulin signalling, thus supporting that ATM play a crucial role in whole body metabolism (Aouadi et al. 2013).

17.2 Monocyte Recruitment and ATM Accumulation

One of the factors contributing to ATM recruitment is thought to be a local increased concentration of free fatty acids in adipose tissue caused by lipolysis in adipocytes, which then act as chemotactic stimuli (Kosteli et al. 2010). However, this recruitment process appears more complex and depends on several chemotactic pathways. Monocyte chemoattractant protein-1 (MCP-1), which is released by adipocytes, endothelial cells and also by the already present ATM, promotes additional monocyte recruitment acting via the MCP-1 receptor, CCR2. Mice deficient in CCR2 display reduced numbers of ATM and reduced production of pro-inflammatory molecules (Weisberg et al. 2006). In line, wild type mice transplanted with CCR2-deficient bone marrow, as well as wild type bone marrow transplanted into MCP-1-deficient mice results in low ATM accumulation (Oh et al. 2012), thus underlining the importance of the MCP-1/CCR2 axis in this process. However, other chemokine systems may also play a role in monocyte recruitment. Whole body CCR5-deficient mice fed a high fat diet as well as mice with specific CCR5-deficiency in bone marrow-derived cells show improved insulin sensitivity and protection from obesity-induced insulin resistance through, at least in part, a reduction in ATM accumulation (Kitade et al. 2012). Another important factor contributing to ATM accumulation is the α4 integrin, a protein that allows monocytes to adhere to the endothelial barrier and their subsequent transmigration. Blockade of α4 integrin signalling by point mutations, resulted in a dramatic reduction of monocyte/macrophage content in adipose tissue. This reduction was due to reduced monocyte/macrophage migration rather than reduced MCP-1 production (Feral et al. 2008).

Metabolic factors also contribute to monocyte infiltration in adipose tissue. As an example, the Clb-associated protein (CAP), which regulates GLUT4 glucose trans-

port in adipocytes, also affects macrophage mobility. CAP-specific deletion in macrophages resulted in a marked reduction in the macrophage content of adipose tissue after either normal chow or high fat diet feeding (Lesniewski et al. 2007). Adenovirus-mediated expression of the Glucagon-like peptide-1 (GLP-1) to obese mice also significantly reduced the adipose tissue macrophage populations (Lee et al. 2012). Factors involved in the control of the metabolic response to fasting and caloric restriction, such as Sirtuin 1 (SirT1), are also involved in ATM accumulation. Adipose tissue SirT1-deficiency triggers inflammation in this tissue by increasing the number of infiltrated ATM (Gillum et al. 2011). These changes were prevented by whole body overexpression of SirT1, suggesting that SirT1 is an upstream regulator of ATM content by controlling pro-inflammatory transcription responses to inducers such as fatty acids, hypoxia and endoplasmic reticulum stress. In line, in human obese subjects SirT1 expression is inversely related to BMI and adipose tissue macrophage infiltration (Gillum et al. 2011).

17.3 Heterogeneity of the ATM Phenotype

ATM present different functional phenotypes depending on the metabolic status (lean or obese), the adipose depot and their location within the depot. ATM from lean mice express many genes characteristic of M2 macrophages (Arg1, IL-10, chitinase 3 and the lectin MGL), which may protect adipocytes from inflammation, while diet-induced obesity leads to the recruitment of $MGL1^{-}CCR2^{+}$ M1 pro-inflammatory macrophages which contribute to the installation of insulin resistance (Lumeng et al. 2007a, b; Fujisaka et al. 2009). The obesity-induced switch of the M2 to M1 phenotype is attributed to a CCR2-dependent monocyte recruitment rather than to the conversion of pre-existing M2 macrophages (Lumeng et al. 2008).

The CD11c surface molecule, which is considered to be an M1 marker, is considerably increased in ATM upon high-fat diet feeding (Lumeng et al. 2007a; Nguyen et al. 2007). Moreover, CD11c is also considered a marker of dendritic cells, whose prototypical function is the stimulation of naïve T cells. However, the absence of T-cell costimulatory receptors and CD1d in murine $CD11c^{-}$ATM define these cells as macrophages rather than dendritic cells (Zeyda et al. 2010). M1 $CD11c^{+}$ macrophages account for the majority of the ATM recruited in obese adipose tissue, in which more than 90 % of the newly recruited monocytes turned in resident $CD11c^{+}$ ATM (Lumeng et al. 2007b, 2008). Interestingly, targeted deletion of adipocytes by apoptosis is sufficient to initiate a large influx of macrophages in fat pads. However, these macrophages display a M2 phenotype, suggesting that living adipocytes are required to initiate or sustain a pro-inflammatory response of the infiltrating macrophages in adipose tissue (Fischer-Posovszky et al. 2011). Within adipose tissue, the major source of IL-4, a potent stimulator of M2 polarization, is the eosinophils. In their absence, the macrophage M2 phenotype is greatly attenuated (Wu et al. 2011).

Comparative studies revealed that the majority of adipose tissue-produced cytokines (TNFα, IL-6), with the exception of leptin and adiponectin, are secreted

by non-adipocyte cells and in particular by M1 polarized macrophages. These pro-inflammatory cytokines may contribute to the low-grade inflammatory state of obesity. Furthermore, while M2 ATM, which express MGL1, are localized in the interstitial space, $MGL1^{-}/CD11c^{+}$ M1 ATM rather surround death adipocytes and are localized in CLS (Lumeng et al. 2008). In obese mice, progressive lipid accumulation in macrophages with age provokes a M2 to M1 switch (Prieur et al. 2011). Lipid accumulation in ATM is associated with an increased $CD11c^{+}$ (M1) and decreased $CD209a^{+}$ (M2) ATM phenotype. However, a unique subclass of lipid-loaded $CD209a^{+}/CD11c^{+}$ ATM has been identified (Prieur et al. 2011). This strict polarization concept has been challenged more recently. Indeed, mouse epididymal ATM recruited in response to a high fat-diet display a mixed M1/M2 phenotype and their transcription profile becomes more M2-like upon extension of diet duration (Shaul et al. 2010). Using MR and CD11c as markers, three distinct ATM populations have been described in gonadal adipose tissue of obese mice (Zeyda et al. 2010). Obesity promotes a shift from a predominant $MR^{+}CD11c^{-}$ quiescent resident macrophage population (expressing low levels of M1 and M2 markers, but high levels of chemokines) to two MR^{-} populations: $MR^{-}CD11c^{+}$ cells exhibiting a M1 inflammatory phenotype and $MR^{-}CD11c^{-}$ cells expressing low levels of inflammatory markers and high levels of M2 markers such as Arg1 and Ym1/2 (Zeyda et al. 2010).

In humans, the amount of ATM correlates with BMI, adipocyte size and total body fat mass (Curat et al. 2006). Fat mass expansion is associated with the accumulation of anti-inflammatory or mixed M1/M2 polarized ATM (Zeyda et al. 2007), characterized by increased MMP activities, indicating a role in tissue remodelling (Bourlier et al. 2008). Human ATM produce the anti-inflammatory cytokines IL-10 and IL-1Ra, but can also secrete pro-inflammatory cytokines such as TNF, IL-6 and IL-1β supporting a role of ATMs in obesity-induced adipocyte dysfunction and metabolic disorders also in man (Zeyda et al. 2007). While the canonical M2 macrophages, characterized as being $CD45^{+}CD14^{+}CD34^{-}MR^{+}$ locate in the peri-endothelium or in interstitial spaces between adipocytes, a population of $CD45^{+}CD14^{+}CD34^{+}MR^{+}$ macrophages (representing approximately 10 % of the M2 population) has been localized in the perivascular region. These macrophages share biological properties (morphology, mesenchymal multipotency) with adipose stem/stromal cells (Eto et al. 2013). Together with increased macrophage numbers, adipose tissue from obese subjects contains increased areas of fibrosis, when compared to lean subjects, which positively correlate with the number of ATM (Spencer et al. 2010). The majority of ATM appears rather associated with fibrosis, than in CLS. While CLS macrophages are predominantly M1, those in fibrotic areas are of the M2c phenotype since they are positive for $CD150^{+}$, a M2c marker (Spencer et al. 2010).

17.4 Inflammatory Responses and Insulin Resistance

Obesity-associated insulin resistance is driven by local adipose tissue inflammation. Pro-inflammatory cytokines (TNFα, IL-1β, IL-8, and IL-6) and other factors such as MCP-1, TGF-β and PAI-1 stimulate inflammatory signalling pathways leading to

the activation of JNK1 and inhibitor of κB kinase β (IKKβ), initiating a cascade towards inflammation-related insulin resistance. Activation of either pathways leads to altered signalling downstream of the insulin receptor, by preventing PI3K activation and by phosphorylating the insulin receptor substrate protein-1 (IRS-1) on inhibitory serine residues thus attenuating insulin action (Gao et al. 2002; Hirosumi et al. 2002). Furthermore, inflammatory cytokines increase SOCS3 expression, thus interfering with insulin receptor (IR) activity (Emanuelli et al. 2000). Adipocyte insulin resistance contributes to reduced insulin-dependent GLUT4 transport leading to an elevation of circulating glucose levels, compensatory secretion of insulin by pancreatic cells precipitating ultimately type 2 diabetes. Using ddY-H mice which spontaneously develop insulin resistance even when fed a standard diet, it has been demonstrated that the infiltration of pro-inflammatory M1 macrophages into adipose tissue precedes the appearance of insulin resistance (Maeda et al. 2013). Results obtained in mice with macrophage-specific deletion of JNK1 or IKKβ demonstrate that obesity does not lead to an impaired insulin action in the absence of the inflammatory component (Arkan et al. 2005; Solinas et al. 2007). Mice with JNK1 or IKKβ-specific deficiency in macrophages become obese when submitted to a high fat diet, but are still protected against insulin resistance. These mice displayed reduced ATM accumulation as well as inflammatory pathway gene expression. Finally, JNK contributes to ATM accumulation, as demonstrated in mice with selective JNK-deficiency in macrophages which are protected against insulin resistance due to the reduced adipose tissue infiltration by macrophages (Han et al. 2013).

Multiple pathways and mechanisms can lead to the propagation of inflammation in obesity, but most of them, if not all, appear related to the final activation of the JNK/IKKβ pathways leading to insulin resistance. Among these pathways, endoplasmic reticulum stress, which can be activated by fatty acids, excess of nutrients and hypoxia, can directly engage the JNK and/or IKKβ pathways (Hummasti and Hotamisligil 2010).

On the other hand, activation of the Toll Like Receptor (TLR)-4 by lipopolysaccharide (LPS) and saturated fatty acids also leads to activation of JNK and IKKβ. Thus, C3H/HeJ mice, which have a loss-of-function mutation in TLR4, are protected against the development of diet-induced obesity (Tsukumo et al. 2007). Besides TLR4, other pattern-recognition receptors, such as the Nlrp3 inflammasome belonging to the family of Nod-like receptors (NLRs), appear involved in obesity-associated inflammation. Ablation of Nlrp3 in mice prevents obesity-induced inflammasome activation in fat depots and enhances insulin signalling. Furthermore, elimination of Nlrp3 in obese mice reduces IL-18 and adipose tissue interferon-γ (IFN-γ) expression, increases naive T and reduces effector T-cell numbers in adipose tissue. Collectively, these data establish that the Nlrp3 inflammasome senses obesity-associated danger signals and contributes to obesity-induced inflammation and insulin resistance (Vandanmagsar et al. 2011). Furthermore, lipids can regulate inflammatory signalling through TLR-independent pathways. Mice over-expressing the diacylglycerol acyl transferase-1 (DGAT-1) in macrophages are protected against diet-induced adipose tissue macrophage accumulation, inflammation and insulin resistance (Koliwad et al. 2010). Finally, activation of the G protein-

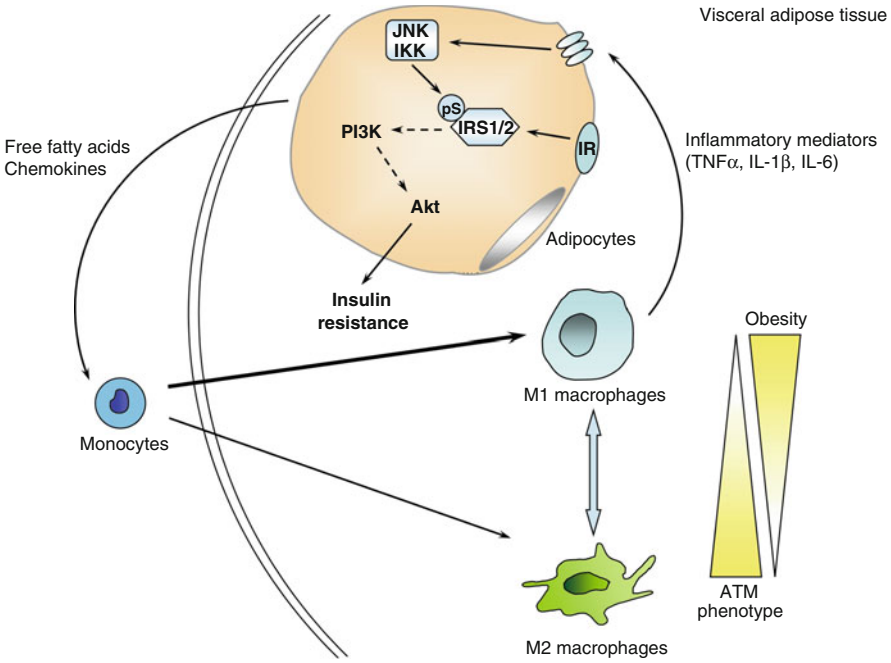

Fig. 17.1 Monocytes are recruited into the adipose tissue, especially in the visceral fat depot, attracted by free fatty acids released by adipocyte lipolysis as well as by chemokines. Once recruited, monocytes differentiate into macrophages, particularly in those with a M1 pro-inflammatory phenotype. The number of M1 macrophages increases with obesity with a parallel reduction of the number of anti-inflammatory M2 macrophages. M1 macrophages secrete pro-inflammatory factors, such as TNFα, IL-1β and IL-6 which interfere with the insulin signalling cascade downstream of the insulin receptor, by preventing PI3K activation and by phosphorylating the insulin receptor substrate protein-1 (IRS-1) on inhibitory serine residues thus attenuating insulin action

coupled receptor GRP120 by omega-3 fatty acids in macrophages reduces the number of inflammatory macrophages in adipose tissue and reverses insulin resistance in obese mice (Oh et al. 2010) (Fig. 17.1).

17.5 Regulation of the ATM Phenotype

Since M1 phenotype ATM are the most important producers of pro-inflammatory cytokines responsible for the development of insulin resistance, studies to determine the molecular mechanisms controlling the balance between the M1 and M2 phenotype as well as its modulation are crucial.

A short term high fat diet challenge promotes alternative polarization of ATM by activating adipose tissue resident Natural Killer T (NKT) cells (Ji et al. 2012a).

In line, activation of NKT cells by α-galactosylceramide enhances alternative M2 macrophage polarization in adipose tissue and improves glucose homeostasis, by a mechanism mediated via the IL-4/STAT-6 pathway (Ji et al. 2012b). Stimulation of obese db/db mice with resolvin D1, a docosahexaenoic acid-derived anti-inflammatory mediator, reduces the number of M1 macrophages resident in CLS and increases the percentage of $MGL1^+$ macrophages (Hellmann et al. 2011). Moreover, resolvin D1 and docosahexaenoic acid switch the ATM toward a M2-like phenotype by increasing the expression of M2 markers (MR, Arg1, IL-10) (Titos et al. 2011). Chemokine pathways also control ATM phenotype polarization: MCP-1-deficiency as well as CCR5-deficiency results in a shift of ATM polarization toward an M2 phenotype (Kitade et al. 2012; Nio et al. 2012). Global as well as hematopoietic cell TLR4-deficiency shifts the ATM phenotype toward an alternatively activated state (Orr et al. 2012). Aging also affects the macrophage phenotype in adipose tissue by decreasing the number of resident M2 ATM, a shift related to decreased expression of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)γ in ATM and alterations in chemokine/chemokine receptor profiles in old mice (Lumeng et al. 2011). Gastric surgery of obese subjects modulates the M1/M2 balance by increasing the number of M2 and decreasing the number of M1 macrophages in subcutaneous adipose tissue (Aron-Wisnewsky et al. 2009). However, weight stabilization by 6 months of a very low calorie diet decreases the amount of ATM, without changing the relative proportion of M1/M2 macrophages (Kovacikova et al. 2011). The discrepancies between these observations can be due to differences in the type of patient population and/or cell surface markers used to identify the ATM sub-populations.

Exercise training also promotes M1 to M2 phenotype switching of ATM in high fat diet-induced obese mice (Kawanishi et al. 2010) and enhances the expression of M2 markers on circulating leukocytes in humans, an effect which may be associated with an increased expression of PPARγ and its cofactors (Yakeu et al. 2010).

Transcription factor networks are master regulators of macrophage polarization. As already mentioned, PPARγ appears a crucial driver of macrophage polarization. Disruption of PPARγ in myeloid cells impairs alternative macrophage differentiation, an effect associated with the development of diet-induced obesity, insulin resistance and glucose intolerance (Odegaard et al. 2007). Myeloid PPARβ/δ-deficiency rendered macrophages unable to shift toward an alternative phenotype, causing inflammation and metabolic perturbations in adipocytes and hepatocytes leading to insulin resistance, increased adipocyte lipolysis and hepatosteatosis (Kang et al. 2008; Odegaard et al. 2008).

In obese mice, treatment with the PPARγ ligand rosiglitazone promotes lipid redistribution from macrophages towards adipocytes, an effect associated with the restoration of a M2 phenotype (Prieur et al. 2011). In line, treatment with PPARγ ligands decreases the number of M1 macrophages in visceral adipose tissue (Fujisaka et al. 2011) and enhances the expression of M2 markers associated with improved insulin sensitivity (Shaul et al. 2010; Stienstra et al. 2008). Krüppel-like factors (KLFs) are a subfamily of the zinc finger DNA-binding transcription factors. Mice with KLF4-specific deletion in macrophages display an enhanced M1 ATM infiltration as well as a shift in the expression of M1/M2 markers in the SVF toward

an M1 phenotype under high fat diet (Liao et al. 2011). Cell cycle regulators can also influence the ATM phenotype. Compared to vascular macrophages, ATM from obese subjects express low levels of the tumour suppressor p16(INK4a) (Fuentes et al. 2011). Moreover, silencing of p16(INK4a) in vascular macrophages increases the expression of M2 markers and enhances the response to LPS, thus giving a phenotype resembling that of ATM (Fuentes et al. 2011).

17.6 ATM: A Possible Link with Cancer Development in Obese Subjects?

Besides their well-established role in the maintenance of a low-grade inflammatory status within adipose tissue, ATM can also affect potential patho-physiological processes. There are now strong epidemiological evidences establishing obesity as a risk factor for the development of cancer, such as colon, breast, oesophagus, kidney, liver and pancreas cancer (Calle and Kaaks 2004). Whereas weight gain is accompanied by higher cancer incidence rates, recent longitudinal studies after bariatric surgery indicate that weight loss decreases cancer rates (Adams and Hunt 2009). During obesity, the expanded AT could contribute to cancer development via a deregulated secretion of pro-inflammatory cytokines, chemokines and adipokines (Prieto-Hontoria et al. 1807). Conditioned medium from ATM, but not from autologous monocyte-derived macrophages (MDM) isolated from morbidly obese patients, induced phenotypic changes and activation of human cancer cells (Mayi et al. 2012). Global gene expression analysis performed on ATM and MDM isolated from the same morbidly obese patients identified several genes over-represented in ATM, compared to MDM, belonging to cytokine–cytokine receptor interaction pathways (including chemokine, hematopoietin, PDGF, interleukin, TNF, interferon and TGFβ pathways), thus resembling tumour-associated macrophages (TAM) (Solinas et al. 2010; Ono 2008). In line, ATM produce growth factors, cytokines, chemokines and proteolytic enzymes involved in the regulation of tumour growth, angiogenesis, invasion and/or promotion of cancer metastasis, as observed in TAM (Mayi et al. 2012; Mantovani and Sica 2010). These data indicate that ATM may contribute to cancer initiation and progression in obese subjects.

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Chapter 18
Macrophages Govern the Progression and Termination of Inflammation in Atherosclerosis and Metabolic Diseases

Gabrielle Fredman and Ira Tabas

18.1 Introduction

Atherosclerosis is a disease of the vasculature and involves an expansion of the arterial intima with lipids, inflammatory cells, and extracellular matrix. While this process rarely leads to clinical symptoms due to preservation of the arterial lumen, a few of these lesions undergo necrotic breakdown, which precipitates acute, occlusive lumenal thrombosis leading to myocardial infarction and stroke (Virmani et al. 2002). In this regard, atherosclerosis underlies the leading cause of death in the industrialized world and identifying mechanisms to curtail disease progression are of global importance.

For many years, atherosclerosis was thought to involve passive lipid deposition in the vessel wall. Today we understand that atherosclerosis is a nonresolving chronic inflammatory disease driven by retention of LPs (Williams and Tabas 1995; Tabas and Glass 2013). It is important to note that inflammation is initially a protective response that involves the activation of immune cells such as macrophages, to eliminate pathogens and repair tissue injury (Majno and Joris 2004). However, an excessive or persistent inflammatory response impairs resolution and leads to chronic inflammation and subsequent tissue damage (Serhan et al. 2007). In atherosclerosis persistent retention of LPs in the subendothelial cell layer of blood vessels potentiates inflammatory processes such as the recruitment of monocytes that give rise to macrophages (Williams and Tabas 1995). Macrophages then become locally deranged and secrete injurious mediators that intensify inflammation (Fig. 18.1). While macrophages are highly regarded as protagonists of atherosclerosis, recently uncovered mechanisms suggest that macrophages can also be key players in the resolution of atherosclerosis and may play a critical role in quelling disease progression (Fig. 18.1).

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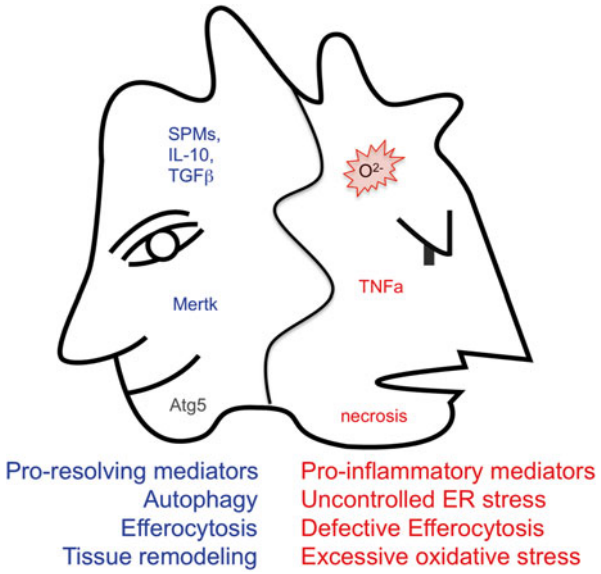

Fig. 18.1 The two-faced macrophage. Macrophages are highly plastic and can modulate their phenotype based on local tissue environments. In the case of nonresolving diseases such as atherosclerosis, persistent external stimuli lead to exacerbated release of pro-inflammatory mediators, excessive oxidative and ER stress responses, and defective efferocytosis. In resolving scenarios, macrophages actively regulate the return to homeostasis by generating and processing pro-resolving mediators and by carrying out efferocytosis and autophagy. Understanding mechanisms to jump-start resolving phenotypes of macrophages should prove helpful in conceiving new strategies to stimulate the resolution of chronic inflammatory diseases such as atherosclerosis

This functional heterogeneity of macrophages may appear paradoxical, but in fact presents an opportunity for therapeutic intervention because it suggests that specific disease-promoting mechanisms can be targeted and those required for normal homeostasis can be spared. This chapter will overview and highlight the complex roles of macrophages in atherosclerosis and provide insight into novel treatment strategies that can harness macrophage function away from inflammation and toward resolution and tissue repair.

18.2 Monocyte Entry into Lesions

Early inflammation caused by retained LPs leads to local activation of endothelial cells and subsequent recruitment of circulating monocytes (Glass and Witztum 2001; Mestas and Ley 2008). Activated endothelial cells secrete chemokines and lipid mediators that interact with select receptors on monocytes to promote their transmigration. Importantly, prevention of monocyte entry by blocking chemokines, lipid mediators or their receptors retards atherogenesis in mouse models of

atherosclerosis (Mestas and Ley 2008; Heller et al. 2005). It should be noted that, although early LP retention precedes and then triggers endothelial activation and monocyte entry, monocyte-derived macrophages in the lesion may subsequently secrete LP-binding proteoglycans (Williams and Tabas 1995) to further amplify LP retention, which can partially explain why the inflammation in atherosclerotic lesions persists and fails to resolve (Tabas 2010a).

Circulating monocytes originate from bone marrow-derived progenitor cells, and this early stage of monocyte development may be regulated by cellular cholesterol content in a manner that can impact atherogenesis. Mice deficient for the cholesterol efflux transporters, ABCA1 and ABCG1, show an increase in circulating monocytes ("monocytosis") and increased atherosclerosis (Yvan-Charvet et al. 2010; Westerterp et al. 2013). Importantly, both the monocytosis and increased atherosclerosis are reversible by restoring cholesterol efflux to high-density lipoprotein (HDL), and there are correlations in humans among high HDL, lower blood monocyte counts, and decreased risk for atherosclerosis (Coller 2005). Further, a known major risk factor for atherosclerosis is diabetes. Recent findings indicate that hyperglycemia causes monocytosis and neutrophilia and impairs atherosclerotic lesion regression (Nagareddy et al. 2013). Briefly, in response to hyperglycemia, neutrophils secrete S100A8/S100A9, which interacts with glucose-inducible RAGE on common myeloid progenitor cells, resulting in the release of inflammatory monocytes from the bone marrow (Nagareddy et al. 2013). Excess circulating activated monocytes result in enhanced and persistent entry into lesions, which impairs regression of atherosclerosis. Interestingly, normalizing blood glucose facilitated resolution by dampening S100A8/S100A9-initiated monocytosis and causing an overall decrease in inflammatory status (Nagareddy et al. 2013). These observations demonstrate a previously unknown crosstalk between diabetic leukocytes and myeloid progenitor cells, leading to increased production of monocytes and neutrophils, and provide a mechanistic link between diabetes and atherosclerosis.

Murine blood contains at least two distinct monocyte subsets, namely Ly6C^{low} and Ly6C^{high} (Woollard and Geissmann 2010). In response to hypercholesterolemia, the bone marrow and spleen overproduce Ly6C^{high} monocytes that enter the circulation, contribute to monocytosis, preferentially accumulate in lesions, and differentiate into macrophages. Ly6C^{low} monocytes are thought to be primarily reparative and to infiltrate atheromata with less frequency. This decreased entry may be due in part to the fact that Ly6C^{low} monocytes have low expression of the chemokine receptor CCR2. Local production of monocyte chemotactic protein-1 (MCP-1) (the ligand for CCR2) is involved in monocyte recruitment to the lesion (Subramanian et al. 2013). Humans also have distinct monocyte subsets, but their properties and functional roles may differ from those of monocyte subsets in mice. Moreover, it is key to understand whether the macrophages that derive from these subsets in mice and humans have functionally distinct roles in atherosclerosis progression (vide infra).

Circulating monocytes first tether along the vascular endothelium via selectins, then become firmly attached through integrins and finally enter the lesions. Briefly, monocytes slowly tether and roll on endothelial cells overlying retained LPs, notably through the interaction of monocyte P-selectin glycoprotein ligand-1 (PSGL-1) with

endothelial selectins (Mestas and Ley 2008). Recent evidence has implicated monocyte type I interferon signaling in the adhesion process (Goossens et al. 2010). Immunohistochemical analysis of human lesions and molecular-genetic causation studies in gene-targeted mice suggest that the monocyte integrins very late antigen-4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1) and the endothelial cell ligands vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) may be particularly important in early atherogenesis because of their role in facilitating monocyte extravasation into lesions. Moreover, it should be noted that activated platelets overlying atherosclerotic lesions might also promote monocyte–endothelial interaction by activating NF-κB signaling and adhesion molecule expression on activated endothelium (Mestas and Ley 2008; Koenen et al. 2009). Integrin-mediated firm adhesion of monocytes is followed by their entry into the subendothelial space (diapedesis) (Kamei and Carman 2010) where they rapidly differentiate into macrophages.

18.3 Macrophages Phenotypes in Lesions

Macrophages display remarkable plasticity and can change their physiology in response to local environmental cues (Mosser and Edwards 2008), as illustrated by the M1 or M2 (Lawrence and Natoli 2011) phenotypes and, more recently, the resolving macrophage (rM) phenotype (Stables et al. 2011). Briefly, macrophages that promote inflammation are referred to as classically activated, or M1, macrophages, and those that promote resolution are known as alternatively activated, or M2, macrophages (Martinez et al. 2009). Thus, an imbalance in the ratio of classically activated and alternatively activated macrophages in atherosclerosis may cause or at least reflect impaired resolution (Tabas 2010a). There are several known factors that can shift the balance in favor of M2 macrophages, including Th2 cell-secreted molecules, such as interleukin-4 (Martinez et al. 2009); the transcription factors peroxisome proliferators-activated receptors (PPARs) γ and δ (Li et al. 2004); and sphingosine-1-phosphate (Nofer et al. 2007). Studies in mice have shown processes that promote Th2 cell polarization (Laurat et al. 2001), activate PPARγ (Chawla et al. 2001), or activate sphingosine-1-phosphate signaling (Nofer et al. 2007) have beneficial effects on atherosclerosis. The degree to which these results reflect M2 polarization or other aspects of resolution in atherogenesis, however, remains to be fully explored. Recently, macrophages from regressing lesions were characterized by enhanced expression of genes associated with an anti-inflammatory M2 macrophage phenotype, including arginase I, CD163, and the C-lectin receptor (Feig et al. 2012). Also, these macrophages preferentially expressed genes that reduced cellular adhesion, enhanced cellular motility, and overall acted to suppress inflammation (Feig et al. 2012). While several studies have classified macrophages as M1 or M2, it is equally important to note that macrophage phenotype is a continuum and classical M1, M2, or rM definitions may not be apparent (Mosser and Edwards 2008)

in all time points of disease progression. Since inflammation is initially a protective response, it would not be surprising in certain scenarios if M1 macrophages act in a protective manner. Conversely, it might be harmful for a macrophage to acquire an M2 phenotype when it is needed to promote host defense and inflammation. These concepts are particularly evident in cancer where M1 macrophages are protective and M2 are harmful (Mantovani et al. 2002). Hence, understanding mechanisms to promote or dampen these phenotypes is of utmost clinical importance.

18.4 Macrophage Foam Cells: Friend or Foe?

18.4.1 Formation and Function

Macrophage foam cells are characterized by massive accumulation of lipid and contribute to all stages of atherosclerosis, from the initial development of fatty streaks to the rupture of unstable plaques (Glass and Witztum 2001; Rocha and Libby 2009). Macrophages in atherosclerotic lesions are thought to acquire the foam cell phenotype through the constitutive uptake and degradation of native and modified lipoproteins via scavenger receptors, micropinocytosis, and phagocytosis of lipoprotein aggregates (Goldstein et al. 1979; Krieger and Herz 1994; Miller et al. 2003; Korns et al. 2011). Excess cholesterol delivered to the macrophage by these pathways must be either exported to extracellular acceptors via cholesterol efflux pathways (i.e., ABCA1 and ABCG1) or esterified for storage in cytoplasmic lipid droplets in order to prevent the cytotoxic effects associated with elevated free cholesterol in the endoplasmic reticulum (ER) membrane (Tabas 2010a). In this regard, ingested LP-derived cholesterol has the potential to influence macrophage function (Feng et al. 2003; Tabas 2004). While much of the cholesterol taken up by macrophages is re-esterified by ACAT1 to cholesterol esters and stored in cytoplasmic droplets, under certain conditions unesterified cholesterol can accumulate in the ER membrane. This results in the induction of the unfolded protein response (UPR), which, if prolonged, can ultimately lead to apoptosis. Also, macrophages persistently exposed to atherogenic LPs may have some degree of enrichment of free cholesterol in the plasma membrane, which may enhance inflammatory signaling through clustering-mediated activation of signaling receptors (Yvan-Charvet et al. 2007; Zhu et al. 2008; Tang et al. 2009). In addition, other proatherogenic lipids such as oxysterols and oxidized phospholipids delivered through the uptake of oxidatively modified forms of LPs can induce macrophage apoptosis and eventual necrosis (Seimon et al. 2010a). In summary, newly infiltrated monocytes differentiate into macrophages that recognize and ingest lipids. Ingesting modified LPs, like ingesting microbes, involves scavenging substances initially perceived to be dangerous. After ingestion of LPs macrophages become foam cells, many of which eventually die and contribute to a large lipid core (i.e., a characteristic of lesions most vulnerable to rupture) (Moore and Tabas 2011).

18.4.2 New Function

Surprisingly, recent evidence indicates that cholesterol accumulation in macrophages may be able to suppress, rather than promote, inflammatory gene expression (Spann et al. 2012). These results suggest that foam cell macrophages themselves do not exert a pro-inflammatory phenotype but rather are influenced by the harsh environment created by other lesional inflammatory cells, such as Th1 and Th17 cells (Charo and Taubman 2004; Hansson et al. 2006), and by the uptake of cholesterol crystals (Duewell et al. 2010) and exposure to debris from apoptotic and necrotic cells (Seimon et al. 2010a). While it is true that the local environment can influence macrophages biology, another possible scenario is that ingestion of LPs immunologically disarms the macrophage and thereby creates an immunosuppressive phenotype. This potential disarmament may render the macrophage unable to provide needed protective pro-resolution and tissue restorative responses. Future in vivo studies are needed to understand mechanisms of failed resolution programs in lesional macrophages to fully determine if foam cell macrophages are in fact beneficial or harmful toward disease progression. The following sections will highlight specific mechanisms whereby macrophages can either contribute to the pathogenesis or the resolution of atherosclerosis.

18.5 Local Uncontrolled Inflammation Deranges Tissue Macrophages and Promotes Plaque Vulnerability

A critical feature of plaque vulnerability is the necrotic core that arises from the combination of excessive apoptosis of lesional macrophages and defective phagocytic clearance, or efferocytosis, of the apoptotic macrophages in lesions (Tabas 2010a). Oxidative stress, growth factor deprivation, and death receptor activation by ligands in atherosclerotic lesions may trigger macrophage death. Several studies have explored mechanisms associated with macrophage-induced plaque necrosis, including the detrimental effect of NF-κB suppression (Kanters et al. 2003) and the beneficial effects of interrupting type I interferon (Goossens et al. 2010) or TLR2/4 signaling (Seimon et al. 2010a). Another known mechanism of macrophage cell death is prolonged activation of endoplasmic reticulum (ER) stress pathways, primarily the UPR (Tabas 2010b; Tabas and Ron 2011). In normal physiology, the UPR functions to correct disequilibria in ER function, but when ER stress is prolonged, persistent expression of the UPR effector CHOP can trigger apoptosis (Tabas and Ron 2011). A recent study using $Ldlr^{-/-}$ mice transgenic for a UPR reporter consisting of a fluorescent XBP-1 protein revealed that fluorescent signals increased as a function of time on the atherogenic diet (Thorp et al. 2011). In advanced lesions, the majority of fluorescence was found close to necrotic cores and localized to macrophages, indicating the importance of macrophage ER stress in atherosclerosis development. Mechanistic studies using athero-relevant stimuli

in vitro revealed that prolonged ER stress is responsible for CHOP-mediated macrophage apoptosis through a pathway involving release of ER calcium and activation of CaMKII (Timmins et al. 2009; Li et al. 2009a). In this context, genetic silencing of CHOP was shown to suppress macrophage death both in vitro and in vivo and decrease advanced plaque necrosis in mice (Thorp et al. 2009; Tsukano et al. 2010). Moreover, studies performed on human coronary artery samples clearly indicated a strong correlation among ER stress markers CHOP and GRP78, lesional cell apoptosis, and unstable atherosclerotic plaques, suggesting that ER stress may also be involved in the development of unstable plaques in humans (Myoishi et al. 2007).

A potent inducer of prolonged ER stress in macrophages is insulin resistance. In this regard, advanced plaques of type 2 diabetics are characterized by large necrotic cores independent of lesion size (Tabas et al. 2010). In mice, macrophages with defective insulin signaling have an amplified ER stress-apoptosis response, leading to increased macrophage apoptosis and plaque necrosis (Han et al. 2006). These ER stress-induced macrophage apoptosis pathways likely complement other consequences of diabetes on lesional macrophages, such as dyslipidemia-mediated pro-inflammatory effects, to promote advanced plaque progression (Tabas et al. 2010).

A key concept related to the mechanism and in vivo relevance of ER stress-induced macrophage death is the presence of compensatory cell-survival pathways. In vitro and in vivo data indicate that before macrophages subjected to prolonged ER stress undergo apoptosis, there is activation of several compensatory cell-survival pathways, including those involving NF-κB, Akt, p38α, and autophagy (Tabas 2010b). In cultured macrophages, the onset of apoptosis correlates with eventual suppression of these pathways. In this context, a driver of apoptosis in ER-stressed macrophages is activation of innate immune pattern recognition receptors (PRRs), which may have initially evolved as a host defense response against invasive organisms that require living macrophages to survive (Seimon et al. 2010b). Two major classes of PRRs, scavenger receptors (SRs) and TLRs, have been studied in atherosclerosis and are relevant to apoptosis in ER-stressed macrophages (Moore and Tabas 2011). These pathways trigger apoptosis in ER-stressed macrophages by suppressing compensatory cell-survival pathways that are induced by prolonged ER stress and by stimulating oxidative stress. Importantly, atherosclerotic mouse models lacking two SRs, SR-A and CD36, or two TLRs, TLR4 and TLR2, are partially protected against advanced lesional macrophage death and plaque necrosis. In addition, a lipoprotein in humans called lipoprotein(a) Lp(a), which is highly associated with atherothrombotic vascular disease and a carrier of oxidized phospholipids (oxPLs) (Bergmark et al. 2008), is a potent trigger of apoptosis in ER-stressed macrophages through oxPL-mediated activation of a CD36/TLR2 pathway (Seimon et al. 2010a). Notably, a study in a transgenic rabbit model showed that increased plasma Lp(a) led to a marked increase in plaque necrosis (Sun et al. 2002). Thus, it is evident that macrophages exposed to persistent signals can contribute to the pathogenesis of atherosclerosis (Moore and Tabas 2011). It is important to note that initial recruitment of monocytes/macrophages is likely a response by the host to protect against the deleterious effects of lipoproteins in the vascular wall. The studies summarized in this section focused on the mechanisms of excessive, dysregulated

macrophage behavior. Yet, a critical, under-explored issue involves the mechanisms macrophages use to turn off or counter these excessive signals. The next section will discuss the processes and mediators that macrophages use to prompt resolution, specifically in the context of atherosclerosis and metabolic disease.

18.6 Harnessing Macrophages to Promote Resolution and Quell Inflammation

The ideal outcome of an inflammatory insult is complete tissue resolution (Majno and Joris 2004). Previously it was thought that resolution was a passive process, but it is now appreciated that resolution is an active and highly coordinated process (Serhan et al. 2007) involving a multitude of mediators, receptors, signaling pathways, and consequently cellular functions highlighted below (Tabas and Glass 2013; Serhan et al. 2007; Tabas 2010a).

18.6.1 Efferocytosis Fails in Advanced Atherosclerosis

A key to resolution is the ability of host cells, like macrophages to clear debris or apoptotic cells (i.e., efferocytosis) and repair damage caused by the inciting agents (Savill 1997; Henson et al. 2001). In the intima of early lesions, efferocytosis is rapid and nonphlogistic (Tabas 2010a). However, as lesions mature, there is an accumulation of TUNEL-positive, non-phagocytosed apoptotic cells. Non-cleared apoptotic cells lose membrane integrity and become secondarily necrotic, contributing over time to region of tissue necrosis that destabilizes plaques and is linked to acute myocardial infarction. In advanced atherosclerotic lesions, mice lacking the engulfment receptor MerTK had a defect in macrophage efferocytosis that correlated with an increase in plaque inflammation and plaque necrosis (Thorp et al. 2008; Ait-Oufella et al. 2008). MerTK expression is highly expressed in macrophages (Seitz et al. 2007) and is critical for the clearance of apoptotic cells in advanced atheromata. Interestingly, recent studies (Thorp et al. 2008; Ait-Oufella et al. 2008) indicate that MerTK can become inactivated under some inflammatory conditions. The possibility that MerTK function is defective is intriguing since this molecule undergoes cleavage by one or more plasma membrane sheddases under inflammatory conditions (Sather et al. 2007). The cleavage of MerTK suppresses efferocytosis by both destroying the receptor and by creating soluble Mer, which competes for the efferocytosis bridging molecules Gas6 and Protein S. Importantly, while Mertk cleavage has been identified in advanced plaques (Hurtado et al. 2011), further in vivo mechanistic studies are needed to understand its function in atherosclerosis.

Studies in mouse models of atherosclerosis have revealed roles for several other macrophage efferocytosis receptors (e.g., Lrp1 and transglutaminase-2, TG2) and their ligands (e.g., MFG-E8 and Gas6) in advanced atherosclerosis. As an example, Fazio and colleagues demonstrated that clearance of apoptotic macrophages was significantly reduced in $Lrp1^{-/-}$ lesions relative to controls (Yancey et al. 2010). Compared with wild-type lesions, $Lrp1^{-/-}$ lesions exhibited larger necrotic cores with more dead cells not associated with antibody-stained macrophages (Yancey et al. 2010). Similarly, the cell-surface TG2 receptor is expressed in lesional macrophages and participates in recognition and engulfment of apoptotic cells. $Ldlr^{-/-}$ mice engrafted with $Tg2^{-/-}$ bone marrow cells exhibit larger aortic root lesions and necrotic cores relative to controls (Boisvert et al. 2006). TG2, in cooperation with the avβ3 integrin, can engage lactadherin (MFG-E8)-opsonized apoptotic cells and promote engulfment (Toth et al. 2009). MFG-E8 is expressed in atherosclerotic lesions and promotes efferocytosis in vitro and in vivo. Mallat and colleagues showed that lesions in mice lacking Mfge8 in bone marrow cells were larger, more necrotic, and had increased apoptotic cellular debris (Ait-Oufella et al. 2007).

Defective efferocytosis may also underlie other diseases associated with failed resolution, including insulin resistance and type 2 diabetes (Tabas 2010a). In this regard, impaired efferocytosis in F4/80-positive cells was observed in atherosclerotic lesions of genetically obese obese/$Ldlr^{-/-}$ (Li et al. 2009b). Interestingly, saturated fatty acids (palmitic and stearic) were increased in obese mice relative to endogenous omega-3s and were implicated in defective macrophage efferocytosis. Accordingly, supplementation of omega-3s, EPA and DHA, reversed deficits in macrophage efferocytosis in obese/$Ldlr^{-/-}$mice (Li et al. 2009b). Recent evidence also indicated that saturated fatty acids in obesity alter resolution of acute sterile inflammation by promoting neutrophil survival and decreasing macrophage phagocytosis (Hellmann et al. 2013). Further, db/db mice exerted elevated PGE_2 and PGD_2 in inflammatory exudates during the development of acute peritonitis. Moreover, in isolated macrophages, palmitic acid stimulated cyclooxygenase-2 induction and prostanoid production. Defects in macrophage phagocytosis induced by palmitic acid were mimicked by PGE_2 and PGD_2 and were reversed by cyclooxygenase inhibition (i.e., aspirin treatment) or prostanoid receptor antagonism (Hellmann et al. 2013), suggesting that aspirin or prostanoid receptor antagonism may provide a therapeutic way to prevent defective efferocytosis in obesity and insulin resistance (Hellmann et al. 2013). Along these lines, obese, insulin-resistant mice were shown to exhibit delayed resolution of wound healing and tissue repair responses, which was due in part to impaired efferocytosis (Tang et al. 2013). Hence, understanding ways to enhance efferocytosis is likely to be important as we conceive new strategies to help resolve several disease processes, including atherosclerosis and metabolic diseases. Collectively, these studies highlight that progression of chronic inflammatory diseases could result in part because of altered resolution, and some of the findings implicate possible roles for endogenous proresolving lipid mediator pathways (vide infra).

18.6.2 Autophagy Is Protective in Atherosclerosis

Recent work uncovered a protective role for autophagy in limiting oxidative damage and excessive ER stress responses in lesional macrophages. In this regard, gene targeting an essential component to autophagy (Atg5) increased the susceptibility of macrophages to undergo apoptosis (Liao et al. 2012) and led to increased lesion and necrotic area in advanced atherosclerotic plaques. Further, there was also to defective efferocytosis of apoptotic plaque macrophages (Liao et al. 2012). In this regard, autophagy inhibition impaired efferocytosis of apoptotic macrophages by defective recognition and binding of apoptotic macrophages. These results were consistent with another finding that indicated macrophage specific knockout of Atr5 resulted in worsening of atherosclerosis due to heightened inflammasome activity (Razani et al. 2012). Moreover, classic inflammasome markers were robustly induced in $ATG5^{-/-}$ macrophages, especially when co-incubated with cholesterol crystals (Razani et al. 2012). Further, cholesterol crystals appear to be increased in $ATG5^{-/-}$ macrophage lesions, suggesting a vicious cycle of crystal formation and inflammasome activation in autophagy-deficient plaques. Collectively, these results demonstrate that autophagy becomes dysfunctional in atherosclerosis that promotes atherosclerosis in part through inflammasome hyperactivation. In addition to providing protective responses in atherosclerosis, autophagy also plays a key role in regulating anti-inflammatory processes by limiting NFκB-mediated signaling (Zang et al. 2012). Autophagy deficiency also reduces cholesterol efflux from lipid-loaded macrophages (Ouimet et al. 2011; Mei et al. 2012) and undoubtedly plays protective role in thwarting atherogenesis.

18.6.3 Anti-inflammatory/Pro-resolving Mediators and Targets

Common examples of anti-inflammatory and pro-resolving mediators include IL-10 (IL-10), transforming growth factor-β (TGFβ), a transcriptional factor called liver x receptor (LXR), and specialized pro-resolving mediators (SPMs) including lipoxins, resolvins, protectins, maresins (Mosser and Zhang 2008; Joseph et al. 2003; Serhan et al. 2008).

IL-10 and TGFβ: The major target cells of IL-10 signaling are macrophages, where IL-10 receptor signaling induces the anti-inflammatory molecule suppressor of cytokine signaling 3 (SOCS3) and inhibits the NF-κB pathway (Mosser and Zhang 2008). The net result is suppressed macrophage-mediated activation of inflammatory T cells and decreased production of inflammatory cytokines and matrix metalloproteinases by macrophages (Mosser and Zhang 2008). IL-10 also activates signal transducer and activator of transcription-3 (STAT3), which inhibits endoplasmic reticulum (ER) stress-induced apoptosis in macrophages by inducing cell-survival molecules, and it down-regulates CD36, which triggers apoptosis in ER-stressed macrophages exposed to toll-like receptor 2 (TLR2) ligand (Seimon et al. 2010a; Li et al. 2008). IL-10 is also a potent enhancer of efferocytosis both

in vitro and in vivo (Lingnau et al. 2007). In mouse models of atherosclerosis, genetic targeting of IL-10 worsens atherosclerosis, and genetic or pharmacologic overexpression is beneficial (Pinderski et al. 2002). For example, Western diet-fed $Ldlr^{-/-}$ mice transplanted with IL-10 transgenic bone marrow showed a 47 % decrease in lesion size and a marked decrease in lesion complexity with an 80 % reduction in necrotic core area compared with mice receiving wild-type bone marrow (Pinderski et al. 2002). Moreover, serum IL-10 levels in humans are diminished in subjects with acute coronary syndromes and are inversely correlated with future atherothrombotic events in survivors of myocardial infarction (Seljeflot et al. 2004). Thus, deficiency in the amounts and/or actions of IL-10 may contribute to the defect in inflammation resolution in atherosclerosis.

TGFβ plays key roles in inflammation resolution, including regulation of CD4+CD25+ regulatory T cells and efferocytes (Wahl et al. 2004; Huynh et al. 2002) and stimulation of a protective scar response by inducing collagen production (Frutkin et al. 2009). A defect in this pathway may contribute to a key feature of vulnerable atherosclerotic plaques, namely, thinning of the fibrous cap (Frutkin et al. 2009). When TGFβ signaling was interrupted in $Apoe^{-/-}$ mice by administration of a decoy soluble TGFβ receptor or an anti-TGFβ neutralizing antibody, a vulnerable plaque phenotype was accelerated, with increased inflammation, large necrotic cores, and thin fibrous caps (Mallat et al. 2001; Lutgens et al. 2002). In contrast, transgenic overexpression of TGFβ in $Apoe^{-/-}$ mice stabilized atheromata by decreasing the above detrimental endpoints (Frutkin et al. 2009).

LXRs: Transcription factors known as LXRs have anti-inflammatory effects in macrophages (Joseph et al. 2003; Marathe et al. 2006). The anti-inflammatory mechanisms of LXR include inhibition of NF-κB-mediated gene induction (e.g., IL-6), suppression of antigen-induced T cell proliferation, and a decrease in MMP-9 production (Hong and Tontonoz 2008; Castrillo et al. 2003), which has been shown to promote plaque disruption in the aorta of aged $Apoe^{-/-}$ mice. LXR can also block the induction of cycloxygenase-2 in LPS-activated macrophages in vitro (Castrillo et al. 2003). In terms of atherosclerosis progression in mice, treatment with LXR agonists markedly suppresses lesion progression, even after lesions have become established, and gene targeting of LXR has the opposite effect (Joseph et al. 2002; Bradley et al. 2007). LXR can also induce another feature of inflammation resolution, macrophage egress from atheromata (Feig et al. 2010).

SPMs: Macrophage 12/15-lipoxygenase (12/15-LOX) plays a critical role in lesional inflammation resolution in chow-fed $Apoe^{-/-}$ mice through synthesis of lipoxin A_4 (LXA_4), resolvin D1 (RvD1), and protectin D1 (PD1) (Merched et al. 2008). In particular, 12/15-LOX deficiency promoted lesion formation. In vitro experiments in this study showed that LXA_4, PD1, and RvD1 suppressed atherosclerosis-relevant inflammatory cytokine production by LPS-activated macrophages, and they enhanced the ability of macrophages to carry out efferocytosis (Merched et al. 2008). In fact, a defining feature of SPMs is that they stimulate and enhance efferocytosis in a receptor-dependent manner (reviewed in Serhan and Chiang (2013)). Recently uncovered mechanisms suggest that RvD1 enhances efferocytosis via counter regulating NF-κB signaling (Lee et al. 2013). Moreover, RvD1 and LXA_4 suppressed TNFα-mediated

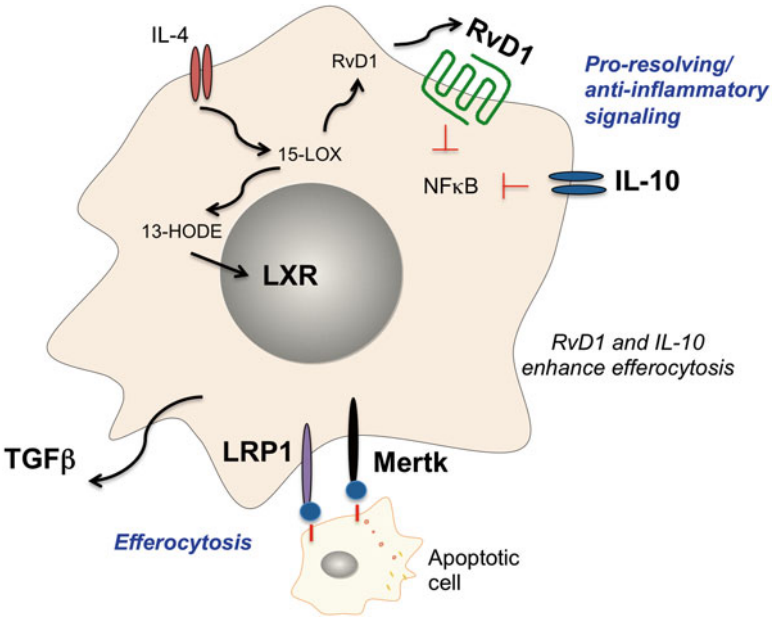

Fig. 18.2 Harnessing pro-resolving mechanisms of macrophages. Several pro-resolving mediators activate macrophage signaling to promote anti-inflammation and tissue repair, including IL-10, RvD1, TGFβ, and LXRs. IL-10 and RvD1 decrease NFκB mediated pro-inflammatory signaling pathways and enhance efferocytosis. Efferocytosis is mediated by receptors including MerTK and LRP-1. 15-LOX is a critical enzyme in regulating pro-resolving responses. For example, IL-4 can increase 15-LOX, which can then generate 13-HODE to activate LXR and subsequent anti-inflammatory signaling pathways (Korns et al. 2011). 15-LOX can also lead to the generation of RvD1 and associated anti-inflammatory and pro-resolving responses (Serhan and Chiang 2013; Serhan et al. 2002). TGFβ can act on neighboring cells to stimulate tissue repair responses to stabilize atherosclerotic lesions

activation of endothelial cells, which plays a critical role in monocyte chemotaxis and adhesion during atherogenesis (Merched et al. 2008). Also, RvD1 suppressed the production of MCP-1 and interleukin-8 and induced anti-inflammatory platelet-derived growth factor-β (PDGFβ); PD1 suppressed MCP-1 and VCAM-1; and LXA_4 suppressed P-selectin (Merched et al. 2008). Consistent with these data, overexpression of 15-LOX in rabbits and subsequent production of LXA_4 was associated with a decrease in high-fat diet-induced atherosclerosis (Serhan et al. 2003). Further, the protective, tissue-restorative role of RvD1 in the vasculature has been demonstrated. Briefly, RvD1 exerted receptor mediated anti-inflammatory actions on smooth muscle cells (SMCs) and prevented excessive SMC migration post balloon angioplasty (Miyahara et al. 2013). SPMs also play protective roles in metabolic disease. Specifically, SPMs protect against liver steatosis and insulin resistance, and they stimulate anti-inflammatory cytokines and protective adipokines (Gonzalez-Periz et al. 2009; Titos et al. 2011; Claria et al. 2012; Hellmann et al. 2011).

Collectively, pro-resolving mediators can link several cellular functions of resolution (Fig. 18.2). For example, IL-10 not only directly blocks inflammatory

responses in M1 macrophages but also stimulates the conversion of M1 macrophages into the M2 subtype and enhances efferocytosis (Martinez et al. 2009). LXR activation in macrophages links three key features of inflammation resolution: suppression of inflammatory cytokine production (Joseph et al. 2003); egress of inflammatory macrophages from atheromata; and enhancement of efferocytosis through the induction of at least two efferocytosis receptors, TG2 and MerTK (Rebe et al. 2009; A-Gonzalez et al. 2009). As a third example of integration, successful efferocytosis leads to the production of TGFβ (Huynh et al. 2002; Frutkin et al. 2009), which stimulates formation of the protective fibrous cap in atheromata (Frutkin et al. 2009) and leads to the generation of SPMs (Schwab et al. 2007; Dalli and Serhan 2012). Together, therapeutic manipulations that affect one or more of these factors may act to curtail progression of atherosclerosis or metabolic disease.

18.7 Concluding Remarks

Macrophages play a key role atherosclerosis. While macrophages evolved to protect the host from pathogenic organisms and to promote tissue repair, macrophages can play maladaptive roles in certain pathologic settings, particularly in those "evolutionary silent" settings that usually occur after reproductive years. Hence, novel therapeutics targeted at enhancing the protective responses of macrophages will be ideal for stimulating resolution in diseases where chronic inflammation underlies the pathology.

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Chapter 19
Myelomonocytic Subsets in Tumor Microenvironment

Jo A. Van Ginderachter

19.1 Introduction

For decades, cancer research was mainly focusing on the discovery of activating (oncogenes) or deactivating (tumor suppressor genes) mutations that transform cells. By now, it has become evident that cancer cell-intrinsic and -extrinsic events equally contribute to cancer progression. As a matter of fact, tumors should be seen as organoid structures in which an intricate interplay exists between cancer cells and stromal cells. These normal tumor-infiltrating cells include fibroblasts, adipocytes, endothelial cells, and cells of the innate and adaptive immune system. Indeed, leukocytes have been reported to play a role in every aspect of cancer, from carcinogenesis and immunoediting to primary tumor growth and metastasis. For example, macrophages are instrumental for maintaining the type of chronic inflammation that predisposes to cancer, such as *Helicobacter pylori*-driven gastric carcinoma (Kaparakis et al. 2008), colitis-associated colon carcinoma (Grip et al. 2003), and hepatitis-mediated hepatocellular carcinoma (Heymann et al. 2009). In established tumors, macrophages contribute to all sorts of tumor-promoting effects, including immune suppression, angiogenesis, increase of cancer cell invasiveness and growth, and the reorganization of the extracellular matrix (Mantovani et al. 1992, 2002; Qian and Pollard 2010).

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19.2 Macrophage Activation States

Macrophages, including tumor-associated macrophages (TAM), are remarkably plastic cells which rapidly adapt to a changing microenvironment (Van Ginderachter et al. 2006b). This capacity forms the basis of the macrophage's polyvalency, whereby these cells are implicated in very diverse functions like tissue development and homeostasis (trophic macrophages), destruction of pathogens, Th1 or Th2-driven inflammation, and wound healing (Gordon and Taylor 2005; Mosser and Edwards 2008).

A commonly used working model for the classification of macrophage activation states is the M1/M2 dichotomy. Classically activated M1 macrophages are induced by Th1 cytokines (IFNγ, TNF) and/or the recognition of endogenous or exogenous danger signals. These cells are prominently pro-inflammatory, initiating and propagating inflammation and mediating pathogen clearance. Conversely, the macrophage phenotype can also be driven by the prototypical Th2 cytokines IL-4 and IL-13, which induce alternatively activated macrophages or M2 (Martinez et al. 2009), often mediated by increased intracellular polyamine levels (Van den Bossche et al. 2012). In addition, multiple anti-inflammatory mediators, including glucocorticoids, TGF-β, IL-10, and apoptotic cells modify macrophage behavior. Consequently, different researchers have proposed different macrophage classification systems (Goerdt and Orfanos 1999; Mantovani et al. 2004; Mosser and Edwards 2008; Martinez et al. 2009). Importantly, all these non-M1 macrophages share the capacity to downregulate Th1 cytokine-driven inflammation, but to promote Th2-dependent pathologies such as asthma and helminth infections, to modulate adaptive immunity and to contribute to wound healing.

However, it should be realized that every classification system has its shortcomings, since macrophages are exposed in vivo to a mixture of stimuli and will adopt mixed functional profiles. This is illustrated by the determination of a consensus gene signature for in vivo induced alternatively activated macrophages, which besides IL-4/IL-13-dependent genes like E-cadherin (Van den Bossche et al. 2009), also encompasses genes that are not induced by any of the known M2-inducing stimuli (Hassanzadeh Ghassabeh et al. 2006).

Notably, in the human situation, M1 and M2 are differentiated from peripheral blood monocytes via GM-CSF or M-CSF, respectively (Puig-Kröger et al. 2009). In this respect, M-CSF-generated macrophages are similar to the trophic macrophages that lack IL-12 and IL-23 secretion but produce high levels of IL-10 (Pollard 2009). This type of macrophage is prominently present in the breast adenocarcinoma tumor microenvironment, whereby M-CSF was reported to upregulate DC-SIGN expression on TAM. DC-SIGN cross-linking induced the secretion of IL-10 and the overall anti-inflammatory phenotype of TAM (Dominguez-Soto et al. 2011). Consequently, an M-CSF response signature in tumors of breast cancer patients correlates with a worse outcome (Beck et al. 2009). At the other end of the spectrum, GM-CSF treatment of mouse mammary tumors reduces tumor progression and metastasis by instructing an antitumoral program in TAM (Eubank et al. 2009). Overall, existing evidence suggests that M1 macrophages are antitumoral, while M2 macrophages rather promote tumor progression.

19.3 Macrophages in Oncogenesis

Meta-analyses have established that patients suffering from chronic infections or autoimmune diseases have a higher risk of developing tumors at the inflammation site. Accordingly, long-term intake of nonsteroidal anti-inflammatory drugs and ligands for the anti-inflammatory nuclear receptor PPARγ provide a chemopreventive effect against cancer development (Grivennikov et al. 2010; Karin et al. 2006; Coussens and Werb 2002; Balkwill et al. 2005; Van Ginderachter et al. 2008). It is clear by now that these drugs mainly target macrophages and that macrophages are central players in inflammation-associated carcinogenesis. For example, blocking the recruitment of macrophages to the inflamed colon by inhibiting the activity of the CCL2 chemokine resulted in a reduced colitis-associated carcinogenesis (Popivanova et al. 2009). CCL2 is also needed to recruit macrophages to the stomach upon *H. pylori* infection, which results in gastric tumorigenesis (Oshima et al. 2011). Similarly, fibroblasts recruit macrophages to the chronically inflamed skin through CCL2, leading to skin carcinogenesis (Zhang et al. 2011). At the inflammatory site, several inflammatory signaling pathways turn macrophages into oncogenic cells. Especially the transcription factor NF-κB appears to be crucial, as illustrated by a reduced tumor formation upon a myeloid-specific deficiency in this pathway (through deletion of IKKβ in these cells) (Greten et al. 2004). On the other hand, a myeloid-specific deficiency in NF-κB antagonizing molecules, such as STAT3 (Yu et al. 2007; Kortylewski et al. 2009), spontaneously triggers colitis under the influence of the normal gut microflora and ultimately leads to colon carcinogenesis (Deng et al. 2010).

Importantly, DEN-mediated hepatocarcinogenesis is a typical example of how sterile damage can lead to cancer. Also in that situation, the development of hepatocellular carcinoma is mediated by macrophages and NF-κB. Mechanistically, macrophages probably become activated by endogenous danger signals (cancer cell necrosis, extracellular matrix degradation) and start to secrete TNFα and IL-6, which drives hepatocyte proliferation and ultimately leads to transformation (Maeda et al. 2005). Accordingly, the Toll-like receptor (TLR) and IL-1R adaptor molecule MyD88, which is upstream of NF-κB activation, is important for carcinogenesis in multiple models (Naugler et al. 2007; Rakoff-Nahoum and Medzhitov 2007; Swann et al. 2008). Notably, macrophages at the tumor site can also become activated by the deposition of immune complexes, resulting from autoantibody production via peripheral B-cell activation and triggering activating FcγRs, at the premalignant site (de Visser et al. 2005; Andreu et al. 2010). Ultimately, inflammatory macrophage-derived molecules stimulating carcinogenesis include prostaglandins (Oshima et al. 1996, 2011); MMP9 (Coussens et al. 2000), reactive nitrogen and oxygen species (Jaiswal et al. 2001; Kim et al. 2003), IL-6 and TNF. In this context, Kupffer cells in males produce higher levels of IL-6 (estrogen downregulates IL-6 production), which is responsible for the higher incidence of hepatocarcinogenesis as compared to females (Naugler et al. 2007). In addition, liver tumor formation is further aided by Kupffer cell-derived TNF triggering NF-κB activation in hepatocytes and activating oval cells (Pikarsky et al. 2004; Knight et al. 2000). Also in gastric and colon

carcinogenesis, a pivotal role for macrophage-derived TNF was demonstrated (Oshima et al. 2011; Popivanova et al. 2008).

In conclusion, convincing evidence exists pointing towards an important role for pro-inflammatory macrophages during carcinogenesis.

19.4 Role of Macrophages in Tumor Immunosurveillance

The finding that mice suffering from immune deficiencies develop more tumors, either spontaneously or following carcinogen treatment (Vesely et al. 2011), has provided a solid experimental basis for the immunosurveillance theory, stating that cancer cells are continuously eliminated by the immune system. It is indeed clear from human patients that the immune composition of tumors (the "immune contexture") is one of the strongest prognostic factors for recurrence and overall survival (Galon et al. 2006; Bindea et al. 2010).

Along this line, macrophages can be treated intratumorally with IFNγ, anti-CD40 or TLR ligands, turning these cells into potent cancer cell killers (Vicetti Miguel et al. 2010; Wu et al. 2009; Beatty et al. 2011; Guiducci et al. 2005). Remarkably, even in patients with pancreatic adenocarcinoma, tumor regression can be obtained by combining the chemotherapeutic gemcitabine with the macrophage activator anti-CD40 (Beatty et al. 2011).

Interestingly, cancer cells appear to be under a selective pressure by macrophages in vivo, suggesting that these cells are natural contributors to immunosurveillance. For example, re-introducing IRF-8 and p53 expression in cancer cells (these genes are often downregulated) leads to macrophage-dependent tumor attack (Greeneltch et al. 2007; Xue et al. 2007). Cancer cells also usually express high levels of the "don't eat me" molecule CD47 (Willingham et al. 2012; Kim et al. 2012), which interacts with the inhibitory receptor SIRPα on macrophages to downregulate phagocytosis (Chao et al. 2010; Jaiswal et al. 2009). Hence, antibody-mediated blockade of CD47 results in cancer cell elimination via two mechanisms: phagocytosis and increased tumor antigen-specific T-cell activation, both of which are mediated by macrophages (Majeti et al. 2009; Tseng et al. 2013). Moreover, CD47 blockade by soluble, high affinity SIRPα variants strongly potentiates the effect of anticancer antibodies through augmenting Fc-mediated phagocytosis (Weiskopf et al. 2013).

19.5 Monocytes and Macrophages in Progressing Tumors

Tumors should be seen as organoid structures, with an extensive and intricate cross-talk between cells in the microenvironment (Egeblad et al. 2010). Such tumor-infiltrating cells comprise a large fraction of mononuclear phagocytes, which are actively recruited to the tumor site and undergo phenotypic changes under the

influence of local stimuli. These myeloid cells are diverse, although the distinct subsets can not always easily be discerned, due to the expression of overlapping markers and the execution of similar functions (Coffelt et al. 2010a; Fig. 19.1).

19.5.1 Monocyte Recruitment to Tumors

Peripheral blood monocytes are recruited to the tumor site from the very early stages of carcinogenesis (Fukuda et al. 2011). Interestingly, cancer growth induces the expansion of hematopoietic progenitor cells and monocytes in the spleen under the influence of angiotensin II, which can readily be recruited to the tumor (Cortez-Retamozo et al. 2013). Several angiogenic and inflammatory mediators such as M-CSF, PDGF, VEGF, and S100A8/A9 have been reported to mediate monocyte recruitment (Ostrand-Rosenberg and Sinha 2009). Moreover, in a mouse ovarian carcinoma model, high levels of intratumoral TNFα stimulate IL-17 production by $CD4^+$ T cells, which in turn attracts myeloid cells to the tumor microenvironment (Charles et al. 2009). A subset of blood monocytes known as Tie2-expressing monocytes (TEM) are oriented near tumor blood vessels under the influence of angiopoietin-2 (Mazzieri et al. 2011), which then increases their immunoregulatory and angiogenic capacity (Coffelt et al. 2010b, 2011).

However, chemokines, which can be expressed in cancer cells under the influence of oncogenes (Borrello et al. 2005), are the best well-described myeloid cell-recruiting molecules. Especially CCL2 is a prominent chemokine in this respect, whereby it influences not only monocyte migration but also function (Ueno et al. 2000). One point of discussion is whether CCL2/CCR2 mainly attracts monocytes to primary tumors or metastases. Qian et al. (2011) report that $Ly6C^{low}$ (patrolling or non-classical) monocytes are the ones accumulating in primary PyMT mouse breast carcinomas, while CCR2 attracts $Ly6C^{high}$ monocytes to pulmonary metastases. In contrast, $Ly6C^{high}$ (inflammatory or classical) monocyte infiltration in primary tumors via CCL2/CCR2 is evidenced in several transplantable models (Movahedi et al. 2012; Hart et al. 2009), in the K14-HPV/E_2 transgenic model of cervical carcinogenesis (Pahler et al. 2008) and the $Kras^{LSL/G12D/+}$; $p53^{fl/fl}$ conditional genetic mouse model of lung adenocarcinoma (Cortez-Retamozo et al. 2012). Even within PyMT primary tumors, CCL2 attracts CCR2-positive monocytes to necrotic regions upon chemotherapeutic injury, which is responsible for tumor recurrence (Nakasone et al. 2012). Hence, it is possible that the extent at which endogenous danger signals are present within tumors determines the type of monocyte attracted. In addition, CCL2 can be nitrated/nitrosylated by reactive nitrogen species inside tumors, producing a chemokine that no longer recruits CTLs but still efficiently attracts immunosuppressive myeloid cells (Molon et al. 2011). Notably, at least part of these myeloid cells originate from the spleen, which functions as a reservoir releasing monocytes upon acute or chronic demand (Cortez-Retamozo et al. 2012). Remarkably, in the absence of monocyte influx (e.g., through CCR2-deficiency), tumors are massively infiltrated by angiogenic neutrophils which sustain or even enhance tumor growth (Pahler et al. 2008; Sawanobori et al. 2008; Movahedi et al. 2012).

19.5.2 Monocyte and Macrophage Diversity in the Tumor Microenvironment

19.5.2.1 Myeloid-Derived Suppressor Cells

"Myeloid-Derived Suppressor Cells (MDSC)" is a functional definition of immature myeloid cells that share the common characteristic of being immunosuppressive. These cells comprise a $CD11b^{+}Ly6C^{hi}Ly6G^{neg}$ monocytic (MO-MDSC) and a $CD11b^{+}Ly6C^{int}Ly6G^{hi}$ granulocytic (PMN-MDSC) population that use different suppressive mechanisms (Van Ginderachter et al. 2006a; Movahedi et al. 2008; Youn et al. 2008). As a matter of fact, several stresses to the body, such as bacterial, fungal, and parasitic infections, trauma and transplantation (Nagaraj et al. 2009; Van Ginderachter et al. 2010) induce the same type of cells. Molecules implicated in tumor-driven MDSC expansion include the transcription factors STAT3 and c/EBPβ and secreted inflammatory mediators like S100A9, prostaglandins, GM-CSF, and IL-6 (Nefedova et al. 2004; Sinha et al. 2007; Cheng et al. 2008; Marigo et al. 2010; Dolcetti et al. 2010; Pylayeva-Gupta et al. 2012; Bayne et al. 2012). Following accumulation, MDSCs need additional triggers that activate STAT1 or NF-κB (Movahedi et al. 2008; Greifenberg et al. 2009) to deploy a multitude of T-cell suppressive mechanisms (Marigo et al. 2008; Ostrand-Rosenberg 2010).

Although CD11b and Gr-1 have traditionally been used as MDSC markers, not all cells co-expressing these molecules are actually immunosuppressive (Greifenberg et al. 2009; Dolcetti et al. 2010). $Ly6C^{hi}MHCII^{neg}$ monocytes, $Ly6C^{hi}MHCII^{hi}$ immature macrophages, $Ly6C^{int}MHCII^{neg}$ immature macrophages, $Ly6C^{int}MHCII^{neg}CCR3^{hi}$ eosinophils, and $Ly6C^{int}MHCII^{neg}Ly6G^{hi}$ neutrophils all fall within this definition and are all present within tumors (Movahedi et al. 2010). HIF-1α expression, under the influence of tumor hypoxia, could be an important mechanism to convey a strongly immunosuppressive phenotype to tumor-associated myeloid cells via the upregulation of iNOS and arginase (Corzo et al. 2010). These intratumoral MDSCs, in particular the monocytic subset, more readily differentiate into macrophages (Corzo et al. 2010), which can be very immunosuppressive as well (Movahedi et al. 2010; Kusmartsev and Gabrilovich 2005). Notably, MDSCs from pancreatic ductal adenocarcinomas are also better suppressors than those from the spleen (Bayne et al. 2012), which could be in agreement with the observation that the suppressive capacity of MDSCs is highest in the inflammatory site (Haverkamp et al. 2011). In addition, splenic and intratumoral MDSC differ in their drug sensitivity, as illustrated by the fact that the STAT-3 inhibitor Sunitinib depletes MDSCs from the spleen but not the tumor. In the tumor microenvironment, high GM-CSF concentrations appear to be responsible for MDSC survival (Ko et al. 2010). To circumvent this problem, 5 fluorouracil has been proposed as a compound that induces MDSC apoptosis both in spleen and tumor, resulting in efficacious anti-tumor immunity (Vincent et al. 2010). Finally, CCDO-Me, a synthetic triterpenoid, does not affect MDSC survival but eliminates their suppressive capacity (Nagaraj et al. 2010).

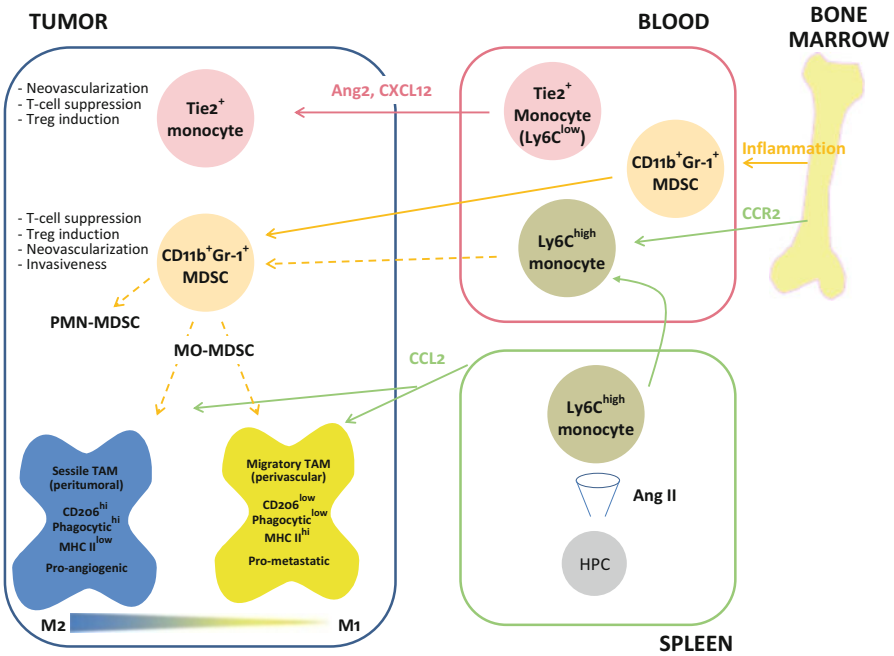

Fig. 19.1 Diversity of monocytes and macrophages in the tumor microenvironment. The main monocyte/macrophage populations found inside tumors include CD11b^{+}Gr-1^{+} MDSC, encompassing a monocytic (MO-MDSC) and a granulocytic (PMN-MDSC) population, Tie2-expressing monocytes and two main TAM subpopulations. The TAMs are typically derived from Ly6C^{hi} monocytes, which are recruited via CCL2 from the bone marrow but also the spleen, where their precursors expand under the influence of angiotensin II. TAM subsets differ at the level of their location and their molecular and functional profile. Each of these tumor-associated myeloid cell types promotes tumor progression via several mechanisms

Other protumoral effects of CD11b^{+}Gr-1^{+} cells have been reported besides immunosuppression. MDSCs (or MDSC-like cells) secrete angiogenic factors such as MMP9 (Yang et al. 2004) and Bv8 (Shojaei et al. 2007a, b). Thus, these angiogenic cells may enter tumors under the influence of CXCL12 and the adhesion molecule VAP-1 (Liu et al. 2010; Marttila-Ichihara et al. 2009) and establish tumor refractoriness to anti-VEGF treatment. In addition, Yang et al. (2008) demonstrated an attraction of CD11b^{+}Gr-1^{+} cells to the invasive edge of mouse mammary carcinomas via CXCL5 and CXCL12, thereby promoting metastasis. Moreover, similar cells move to the premetastatic niche through S100A9, where they prepare the tissue for cancer cell arrival (Hiratsuka et al. 2006; Yan et al. 2010).

19.5.2.2 Tie2-expressing monocytes/macrophages (TEM)

Tie2-expressing monocytes (Tie2 being the angiopoietin-2 receptor) are a relatively minor subset of peripheral blood monocytes with an inherently strong angiogenic potential (De Palma et al. 2005). They are Ly6C^{low} CCR2^{neg} CD62L^{neg} in mouse and CD14^{low}CD16^{hi} in human, thus sharing features with patrolling monocytes (De Palma et al. 2005; Venneri et al. 2007; Pucci et al. 2009). Several pathways of TEM recruitment to the tumor have been observed. Tumor endothelial cells secrete angiopoietin-2 that constitutively attracts TEMs, upregulates their Tie2 expression, and mediates their localization near blood vessels, allowing these cells to play a non-redundant role in tumor neovascularization (Venneri et al. 2007; Murdoch et al. 2007; Coffelt et al. 2010b; Pucci et al. 2009; De Palma et al. 2005; Mazzieri et al. 2011) and Treg induction (Coffelt et al. 2011). However, upon vascular damage CXCL12 is induced in tumors, which recruits TEMs via CXCR4. This mechanism is responsible for the failure of therapies based on vascular-disrupting agents (Welford et al. 2011).

Interestingly, the tumor-infiltrating potential of TEMs has been exploited to deliver therapeutic proteins to the tumor microenvironment. In this context, TEM have been transduced with IFNα and semaphorin 3A, both of which lead to reduced tumor growth and metastasis (De Palma et al. 2008; Casazza et al. 2011).

19.5.2.3 Tumor-associated macrophages (TAM)

It has been realized since quite a while that a high TAM density correlates with a poor prognosis in many different cancer types (Bingle et al. 2002; Lewis and Pollard 2006), and a recent meta-analysis of the literature confirms this link (Zhang et al. 2012). Interestingly, the only notable exception seems to be colon cancer where high TAM levels are favorable (Zhang et al. 2012). In line with these findings, high expression levels of CCL2 and M-CSF, as well as the presence of a M-CSF response gene signature in primary tumors and metastases predict worse outcome (Qian and Pollard 2010; Mantovani and Sica 2010; Sharma et al. 2010; Webster et al. 2010; Espinosa et al. 2009).

Experimental proof for the importance of TAM in tumor progression came from the transgenic MMTV-PyMT model in an M-CSF-deficient background. Although primary tumor growth is unaffected by the macrophage deficiency, progression to invasiveness and metastasis is delayed (Lin et al. 2001). Also the growth of transplantable tumors is suppressed when M-CSF or M-CSFR function is genetically or pharmacologically ablated (Nowicki et al. 1996; Aharinejad et al. 2004; Kubota et al. 2009; Priceman et al. 2010). By now it is clearly established that TAM promote cancer progression via multiple mechanisms [reviewed in Qian and Pollard (2010), Mantovani and Sica (2010)].

TAM Heterogeneity

Different functions for distinct TAM subpopulations have been predicted based on the notion that TAM are found in diverse areas of the tumor (Lewis and Pollard 2006). In PyMT mammary carcinomas, some TAMs are located along blood vessels as single cells or in clusters deep inside the tumor, while other macrophages are rather located at the tumor margins (Wyckoff et al. 2007). The vessel-lining TAMs produce EGF, while cancer cells often secrete M-CSF. As such, these cells co-migrate in a coordinated fashion leading to a more efficient cancer cell intravasation (Wyckoff et al. 2007; Goswami et al. 2005). Remarkably, a similar paracrine loop is initiated by other growth factors like CXCL12 and heregulin-β1 (Hernandez et al. 2009). These data are confirmed by intravital microscopy, following the migration path of photoswitched cancer cells in mammary tumors and showing that cancer cells are only motile in a vascularized environment nearby perivascular macrophages (Kedrin et al. 2008). As a matter of fact, the presence of a "tumor microenvironment of metastasis" (TMEM)—i.e., a tripartite interaction between endothelial cells, macrophages, and invasive cancer cells—in primary breast tumors is predictive of the occurrence of distant metastases (Robinson et al. 2009).

By now, several markers have been described to discern between distinct TAM subpopulations. We studied TAM subsets in several mouse tumor models, either transplantable or transgenic, and found consistently two main populations which differed at the level of MHC II expression (MHC II^{low} versus MHC II^{high}) (Movahedi et al. 2010). Interestingly, these subsets reside in distinct tumor regions, whereby MHC II^{low} TAM are found in the most hypoxic regions (Movahedi et al. 2010, 2012). Moreover, MHC II^{low} TAM are overall more M2 oriented, expressing higher levels of typical M2 markers such as IL-4Rα (CD124), Scavenger receptor-A, stabilin-1, and Macrophage Mannose Receptor (CD206). The latter has been exploited to visualize hypoxic macrophages in vivo. Indeed, nanobodies (i.e., antigen-recognition domains of camelid heavy chain-only antibodies) against MMR readily penetrate tumors, thanks to their small size, and bind MMR^{high} (MHC II^{low}) TAMs (Movahedi et al. 2012). Remark that MMR is also expressed on cells outside of the tumor. However, these extratumoral binding sites can be blocked by a bivalent version of the anti-MMR nanobodies, which has an exceptionally high affinity for its molecular target, but less potently infiltrates tumors because of its larger size. Following such blockade, monovalent radioactively labelled nanobodies primarily go to the tumor and allow molecular imaging of hypoxic macrophages (Movahedi et al. 2012). Notably, hypoxia is not a major driver of the intratumoral differentiation pathway towards MHC II^{low} and MHC II^{high} TAMs, nor does it influence the expression of typical M1/M2 marker genes, but it rather fine-tunes the molecular profile of the MHC II^{low} cells. This includes an upregulation of typical pro-angiogenic genes such as VEGF, leading to an enhanced angiogenic activity (Laoui et al. 2014). Overall, the identification and targeting of markers expressed on hypoxic TAM

could lead to several applications: (1) the visualization of hypoxic regions, for example via molecular imaging using anti-MMR nanobodies (Movahedi et al. 2012), (2) coupling of such nanobodies or other targeting agents to therapeutic isotopes, allowing the administration of high energy radio-emitters specifically to the relatively radioresistant hypoxic regions, (3) coupling of such targeting agents to toxic compounds may allow the elimination of tumor-promoting hypoxic TAMs. As a matter of fact, recent evidence highlights the fact that MHC II^{low} TAMs indeed seem to be more tumor-promoting via the production of immunosuppressive cytokines (IL-10, TGF-β) and angiogenic molecules (VEGF, MMP9), while MHC II^{hi} TAMs exert antitumoral functions (Wang et al. 2011). The question remains to what extent these findings correlate with the human situation. MHC II^{high} and MHC II^{low} TAM populations are also found in distinct regions of human hepatocellular carcinomas, the latter of which secreting higher levels of IL-10, suggestive of a more M2-like phenotype (Kuang et al. 2007). However, in human liver tumors, MHC II^{high} TAMs (or monocytes) are found in peritumoral regions, express high levels of PD-L1 which mediates T-cell suppression (Kuang et al. 2009), and stimulate Th17 expansion fostering angiogenesis (Kuang et al. 2010). Hence, at least in this tumor type, MHC II^{hi} TAM appear to be strongly tumor-promoting.

Further evidence for the existence of distinct TAM subsets comes from cutting-edge microscopy. For example, via spinning disk confocal microscopy CSF-1R^{+}CD68^{+}CD206^{+} phagocytic cells (dextran uptake) were identified as sessile cells at the tumor border of mouse mammary carcinomas, while CD68^{+}CD206^{neg} non-phagocytic myeloid cells (monocytes?) were migratory (Egeblad et al. 2008). Along the same line, LYVE-1^{+} macrophages localized at the periphery of xenotransplanted human mammary carcinomas, while LYVE-1^{-} macrophages were found within the tumor mass (Iyer et al. 2012). Since both CD206 and LYVE-1 are M2-associated markers, it appears that this type of macrophage accumulates at the tumor edges without displaying great motility. This notion is even further strengthened by gene expression analyses, comparing TAMs that co-migrate with cancer cells in an in vivo migration assay with more sessile phagocytic TAMs (Ojalvo et al. 2010). Again, dextran^{+} sessile TAM express higher levels of all M2 signature genes (Hassanzadeh Ghassabeh et al. 2006), including CD206, as compared to the migratory population.

The exact pathways regulating the differentiation/activation of these distinct TAM subsets remain unknown so far. The Ets2 transcription factor could be one mediator, since an Ets2-driven transcriptional program in TAM promotes metastasis and hence could possibly be linked to the pro-metastatic TAM subset (Zabuawala et al. 2010). In any case, the available evidence from independent labs suggests the existence of at least two main TAM populations: (1) M-CSFR^{+}Gr-1^{neg} Dextran^{neg}CD206^{neg}MHC II^{high} migratory TAM in the vicinity of vessels and probably located more inside the tumor. These cells are less M2-polarized and support cancer cell migration and intravasation, (2) M-CSFR^{+}Gr-1^{neg}Dextran^{+}CD206^{+}MHC II^{low} TAM that remain sessile at tumor margins and/or hypoxic regions. These cells are more M2-like or "trophic" in nature. Although an apparently straightforward dichotomy at first, some cells might complicate matters. In particular, TEM have been shown to stay attached to blood vessels in the presence of high angiopoietin-2 and Tie2 levels (Mazzieri et al. 2011). However, TEM are rather M2-oriented and express high levels of CD206 at their surface (Pucci et al. 2009).

As a final remark, the discovery of distinct TAM subsets could explain earlier findings showing a mixed M1/M2 gene expression profile for unseparated TAMs (Biswas et al. 2006; Umemura et al. 2008; Doedens et al. 2010). Indeed, distinct TAM subsets present within the total TAM population probably contribute different types of genes to the expression profile.

19.6 Concluding Remarks

It is clear by now that mononuclear phagocytes in the tumor microenvironment actively contribute to tumor progression and hence should be considered as valuable targets for therapeutic intervention. Current attempts aim to repolarize TAM into antitumoral effector cells (Guiducci et al. 2005; Stout et al. 2009; Beatty et al. 2011) or to eliminate TAM (Luo et al. 2006; Song et al. 2009). Progress in this field will greatly benefit from the identification of additional signaling pathways and molecules that regulate the macrophage phenotype at the tumor site and are responsible for their tumor-promoting functions. In addition, a further fine-tuning of TAM heterogeneity will lead to a better appreciation of the existence of specialized TAM subsets and might allow a more targeted approach, whereby tumor-promoting macrophages are inhibited while antitumoral cells are maintained or even stimulated.

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Chapter 20
Tumor-Associated Macrophages

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20.1 Origin of Tumor-Associated Macrophages

Macrophages infiltrate neoplastic lesions since the early stages of tumorigenesis and usually precede lymphocytes (Mantovani et al. 1992; Clark et al. 2007). TAM derive from hematopoietic monocytic precursors circulating in the blood. In mice, TAM progenitors are essentially $CD11b^+$ $Ly6C^+$ $CCR2^+$ monocytes (Qian et al. 2011). Tumors frequently produce hematopoietic growth factors (M-G-and GM-CSF, as well as IL-3 and Angiotensin II) which stimulate bone marrow myeloid progenitors, increasing the production of monocytes/macrophages (Cortez-Retamozo et al. 2013; Pollard 2009). A recent paper described that during tumor progression myeloid progenitors expand in the spleen of tumor-bearing mice and that TAM can directly originate from spleen-borne myeloid cells (Cortez-Retamozo et al. 2012). Macrophage proliferation in peripheral tissues has also been identified as a determinant of macrophage accumulation, especially at sites of M2-polarized inflammation (Jenkins et al. 2011). However, the proliferating potential of differentiated macrophages is very limited, and TAM mostly rely on blood monocytes for their accumulation.

Monocyte recruitment in tumors is regulated by a number of chemotactic factors, especially chemokines of the CC family. For example, the chemokine CCL2 was discovered as a tumor-derived factor inducing chemotaxis in blood monocytes (Bottazzi et al. 1983; Zachariae et al. 1990). Tumor and stromal cells secrete also

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other chemoattractants, such as colony-stimulating factors (GM-CSF and M-CSF), VEGF, and TGF-β (Mantovani et al. 2006; Pollard 2004).

Once in tumors, monocytes differentiate to mature macrophages primarily because of the presence of locally produced M-CSF. Human tumors secrete M-CSF and its production correlates with poor prognosis in ovarian, breast, and endometrial cancer (DeNardo et al. 2011).

Mononuclear phagocytes are versatile cells capable of displaying different functional activities, some of which are antagonistic: they can be immuno-stimulatory or immuno-suppressive, and either promote or restrain inflammation (Martinez et al. 2009; Gordon and Taylor 2005; Auffray et al. 2009). This functional plasticity is regulated by local cues to which they respond. For instance, during bacterial infections macrophages first orchestrate the acute inflammatory response to eliminate invading pathogens; at later times they transform in scavengers of tissue debris; further on, they trigger the proliferative phase of healing by releasing a variety of growth factors and cytokines which recruit and activate fibroblasts and new vessels (Auffray et al. 2009; Gordon 2003). This functional heterogeneity has been simplified in the macrophage polarization concept where the two extreme phenotypes, the M1 and M2 macrophages, have distinct features (Gordon and Taylor 2005; Allavena et al. 2008; Van Ginderachter et al. 2006). M1 or classically activated macrophages are stimulated by bacterial products and Th1 cytokines (e.g. IFNγ); they are potent effectors that produce inflammatory and immunostimulating cytokines to elicit the adaptive immune response, secrete reactive oxygen species (ROS) and nitrogen intermediates and may have cytotoxic activity to transformed cells. M2 or alternatively activated macrophages differentiate in microenvironments rich in Th2 cytokines (e.g., IL-4, IL-13); they have high scavenging activity, produce several growth factors that activate the process of tissue repair and suppress adaptive immune responses (Gordon and Taylor 2005; Allavena et al. 2008).

Conditioned by the local milieu, monocytes differentiate as tumor-educated macrophages. Factors such as M-CSF, PGE2, TGF-β, IL-6, IL-10, and prostaglandins have the potential to modulate and polarize monocytes mainly into immune-suppressive and pro-tumoral effectors or M2-like macrophages. In molecular profiling studies, murine TAM mostly display hallmarks of M2 macrophages: *arginase-I*, *YM1*, *FIZZ1*, *MGL2*, *VEGF* and *MMPs*, as well as an immunosuppressive phenotype: high IL-10 and TGFβ and low IL-12, RNI and MHC II, which functionally correlate with reduced cytotoxicity and antigen-presenting capacity (Biswas et al. 2006; Ojalvo et al. 2010). Similar findings were observed in human TAM from ovarian cancer patients (Solinas et al. 2010; Mayi et al. 2012). When the RNA profile of human TAM was compared with that of in vitro polarized M1 and M2 macrophages it was observed that several upregulated genes in TAM (e.g., *osteopontin*, *fibronectin*, *scavenger* and *mannose receptor*) were similarly upregulated in M2 macrophages. Indeed, by the Principal Component Analysis, the global profiling of TAM fell close to that of M2-polarized macrophages (Solinas et al. 2010).

TAM heterogeneity has now emerged, depending on tumor type, disease stage (early vs late), tumor location (hypoxia vs normoxia), or other microenvironmental cues (Biswas et al. 2006; Qian and Pollard 2010; Movahedi et al. 2010; Lewis and

Pollard 2006). Differential expression of specific markers (e.g., CD206, CD163, MHC II, Ly6C, CCR2) or signatures from transcriptional profiling have been used to define TAM subsets (Movahedi et al. 2010; Gabrilovich et al. 2012; Ojalvo et al. 2009). Notably, murine TAM from fibrosarcoma showed also the expression of typical M1 factors such as IFN-inducible chemokines (CCL5, CXCL9, CXCL10, CXCL16) (Biswas et al. 2006).

20.2 Pro-tumor Functions of TAM

20.2.1 Tumor Cell Proliferation and Survival

TAM and related myeloid cells (MDSC and Tie2$^+$ monocytes) modulate different aspects of tumor cell biology, from cell proliferation and survival to invasion and metastasis (Fig. 20.1).

Previous and more recent studies demonstrated that experimental tumors have slower progression and dissemination in macrophage-depleted animals (Lin et al.

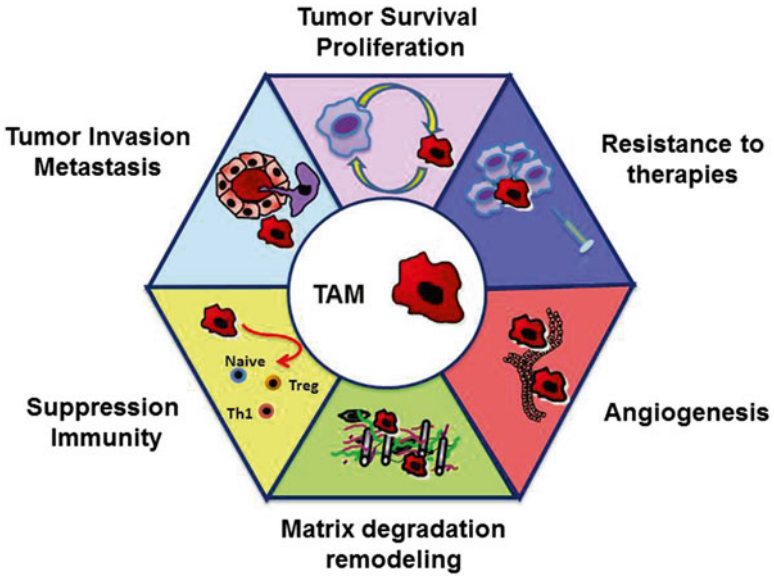

Fig. 20.1 Tumor-associated macrophages (TAM) display several pro-tumor functions. TAM promote the survival of neoplastic cells from apoptotic stimuli and their proliferation, by producing several growth factors and cytokines (e.g., EGF, IL-6), and tumor angiogenesis, via release of VEGF and other angiogenic factors. TAM have an intense proteolytic activity and degrade the extracellular matrix, thus favoring tumor cell intravasation and dissemination to distant sites. TAM have immune suppressive functions and inhibit adaptive Th1-oriented antitumor immune responses, overall contributing to resistance to therapies

2001). More recent evidence has been provided that tumor macrophages sustain the survival also of cancer stem or tumor initiating cells, a phenomenon which obviously impacts on tumor evolution and resistance to therapies (Mitchem et al. 2013; Yang et al. 2013; Jinushi et al. 2011). In vivo imaging experiments demonstrated that TAM within tumors extend cytoplasmic protrusions for prolonged physical interaction with cancer cells (Leimgruber et al. 2009). Direct effects on tumor proliferation are mediated via the production of trophic and growth factors such as members of EGF, FGF, PDGF families. A paracrine interaction has been described where tumor cells recruit and sustain macrophage viability by releasing M-CSF and TAM, in turn, produce EGF (Pollard 2004; Mantovani et al. 2008; Ingman et al. 2006). Matrix-bound growth factors are also released in active form upon ECM degradation, actively mediated by TAM (Joyce and Pollard 2009; Hynes 2009). Cytokines produced by TAM, such as TNF and IL-6, play a major role in tumor growth; TNF triggers the transcription factor NF-κB in tumor cells, and consequently activates a survival programme; IL-6 activates the STAT3 pathway inducing the expression of genes important for cell cycle progression (such as cyclin D and PCNA) and suppression of apoptosis (Bcl-XL, Bcl-2, and Mcl-1) (Li et al. 2011). In mouse models of pancreatic and colon cancers, the source of IL-6 was mainly tracked in macrophage (Grivennikov et al. 2009; Bollrath et al. 2009).

Factors released by tumor cells, including matrix proteins, cytokines, enzymes, and coagulation factors, may reach distant sites via systemic circulation and prepare the soil of a pre-metastatic niche. Macrophages and other myeloid cells are recruited in these niches and actively co-operate to the survival of disseminating cells in an environment otherwise potentially hostile (Psaila and Lyden 2009; Gil-Bernabe et al. 2012).

20.2.2 Matrix Remodelling, Tumor Invasion, and Metastases

In normal tissues, resident stromal cells (fibroblasts, leukocytes, endothelial cells) are typically quiescent and the matrix functions to cooperatively integrate complex signalling between these components. This keeps under strict control various cellular processes such as growth, death, adhesion, migration, gene expression and differentiation, and are of relevance either to maintain homeostasis and to manage with tissue repair in case of injury (Hynes 2009; Barkan et al. 2010). The tumor stroma, instead, is characterized by a remarkable subversion of the tissue architecture, especially in poorly differentiated carcinoma, and contains activated fibroblasts and leukocytes, and irregular vessels (Iozzo 2005; Egeblad et al. 2010). Furthermore, ultra-structural and immunohistochemical analyses revealed a different composition of some ECM proteins (e.g., tenascin, decorin, byglican, alpha smooth muscle actin, fibulin-1, fibronectin), and the appearance of spliced protein isoforms that are not normally expressed (Hynes 2009; Pupa et al. 2002).

TAM produce and secrete several different ECM proteins. The gene expression profile of TAM isolated from human tumors revealed the up-regulation of several genes coding for different matrix proteins, among which are: osteopontin, osteoactivin, various collagens, fibronectin, and a truncated isoform of fibronectin termed migration stimulation factor (Solinas et al. 2010). Altered expression of ECM-related genes by myeloid or other stromal cells has been studied in association with patient clinical outcome (Ramaswamy et al. 2003; Chang et al. 2005; Chibon et al. 2010). The expression of VEGF and MMP7 predicted the risk of poor prognosis in hepatocellular carcinoma (Gao et al. 2011). In stomach cancer, the transition from pre-invasive to invasive lesions was characterized by the up-regulation of TGF-related genes (*thrombospondin 1*); metalloproteases (*MMP1*); *junction-mediating and regulatory protein* (*JMY*); markers of stromal activation: *fibroblast activation protein alpha* (*Fap-α*) (Saadi et al. 2010). In diffuse large-B-cell lymphoma, a stromal signature encoding ECM components was more highly expressed in the non-malignant fraction and included: fibronectin, SPARC, collagen, and laminin isoforms.

In addition to secreting a number of ECM proteins, macrophages produce several proteolytic enzymes. Indeed, even if neoplastic cells and fibroblasts are strong producers, TAM are considered the major cell type expressing protease activity in tumor tissues (Joyce and Pollard 2009; Kessenbrock et al. 2010).

Matricellular proteins are degraded by specific proteases which can be grouped in large families and include not only matrix metalloproteases (MMPs), cathepsins, hyaluronidases, and ADAM proteases, but also heparinase, elastase, urokinase-type plasminogen activator (uPA), plasmin, and others (Mott and Werb 2004). Gene profiling of TAM isolated from human ovarian carcinoma revealed that among the most upregulated genes were several proteolytic enzymes: MMPs (12, 9, 1 and 14), Cathepsins (L, C, Z and B), uPA, lysosomal enzymes, and ADAM proteases (Solinas et al. 2010).

Proteolytic degradation alters stroma stiffness and removes the physical barriers between cells, facilitating the invasion of migrating cells (neoplastic and endothelial cells). Disruption of integrin-mediated anchorage and focal adhesion kinase (FAK) is pivotal for cancer cell invasion into the adjacent spaces (Joyce and Pollard 2009; Fashena and Thomas 2000; Desgrosellier and Cheresh 2010). It has been demonstrated that the cathepsin protease activity of IL-4-stimulated TAM promotes tumor dissemination (Gocheva et al. 2010). IL-4 is produced by tumor-infiltrating CD4 T-cells and there is mounting evidence of its relevance in the polarization of macrophages with pro-tumor functions (DeNardo et al. 2009; Wang and Joyce 2010).

Cleavage of matrix molecules also reveals available binding sites that were previously masked to cell surface receptors: for example, MMP-2 degradation of collagen unveils integrin-binding sites that rescue melanoma cells from apoptosis (Pupa et al. 2002). Cryptic epitopes of fibronectin trigger angiogenesis and tumor growth (Pupa et al. 2002). Over the last decade there has been recognition that ECM fragments can modulate multiple functions of innate immune cells, via the activation of Toll-like receptors. For instance, versican activates TLR2 and TLR6 on TAM and stimulates the production of IL-6 and TNF, two prototypic cytokines of

cancer-related inflammation (Kim et al. 2009). Hyaluronan fragments induce the expression of inflammatory genes in immune cells through activation of TLR4 and TLR2 as well as the CD44 receptor (Jiang et al. 2005).

The matrix is also a repository of growth factors: EGF, FGF, and TGFβ family members, as well as PDGF and VEGF, bind to various ECM components and are stored in an inactive form. The increased proteolytic activity releases active growth factors which stimulate tumor as well as stromal cells (Hynes 2009; Pupa et al. 2002). For example, MMP-3 cleaves decorin, delivering active TGF-beta; MMPs, plasmin and heparinase unleash the angiogenic factor FGF-beta; MMP13 releases VEGF (Lederle et al. 2010). Of note, ECM fragments with angiostatic activity can also be generated; thus, neo-angiogenesis really depends on the balance between pro-and anti-angiogenic factors.

TAM and their released factors (e.g., IL-1 and TNF) have long been known to augment tumor metastasis (Apte et al. 2006; Giavazzi et al. 1990; Balkwill 2009). The direct role of TAM in cancer cell invasion has been visualized in experimental tumors in vivo by multiphoton microscopy: by using fluorescently labelled cells Wyckoff and colleagues showed that tumor cell intravasation occurs next to perivascular macrophages (Joyce and Pollard 2009; Wyckoff et al. 2007; Richards et al. 2013). Further support to the concept of a reciprocal interaction between tumor cells and TAM was provided by a study where SNAIL-expressing keratinocytes became locally invasive after macrophage recruitment elicited by M-CSF (Du et al. 2010).

A recent study demonstrated the importance of TGFβ signalling in tumor-associated myeloid cells is also important for their promoting effect on metastases. Mice with deletion of Tgfbr2 specifically in myeloid cells had less metastasis. TGFβ signalling promotes an immune suppressive phenotype and inhibition of adaptive immunity (Pang et al. 2013). Another study reported that TAM play a relevant role in EMT-promoted tumor progression via paracrine production of TGF-β. Immunohistochemical analysis of tumor samples from NSCLC patients identified a positive correlation between intratumoral macrophages, EMT markers, intraepithelial TGF-β levels, and tumor grade (Bonde et al. 2012).

20.2.3 Hypoxia/Angiogenesis

Tumor hypoxia develops as a result of an imbalance between oxygen supply and consumption in proliferating tumors. As a consequence, pO_2 levels fall and hypoxia arises, thus promoting a process of angiogenesis, the generation of new blood vessels supporting tumor metabolic needs (Folkman 2002). In addition, intratumor hypoxia is associated with a malignant phenotype characterized by uncontrolled tumor growth and increased risk of metastasis (Vaupel and Mayer 2007; Sullivan and Graham 2007). Normal cells can adapt their metabolism to environmental pO_2, whereas tumor cells always privilege glycolysis regardless of oxygen availability (Warburg effect) (Warburg 1956). The gene responsible for this "aerobic glycolisis"

is the pyruvate kinase isoenzyme type M2 (M2-PK), an HIF-dependent gene, and the up-regulation of M2-PK is explainable by oncogene-mediated, hypoxia-independent HIF-1 stabilization (Mazurek et al. 2005).

Macrophages preferentially accumulate in the poorly vascularized regions of tumors and respond to the levels of hypoxia with a transcription program in which mitogenic, pro-invasive, pro-angiogenic, and pro-metastatic genes are upregulated (Mantovani and Sica 2010). The hypoxia-inducible factor (HIF) pathway is essential for the recruitment and activation of TAMs into solid tumors, and contributes to shaping their pro-tumor functions (Cramer et al. 2003). We reported that hypoxic induction of HIF-1α in TAMs influences the positioning and function of tumor cells, stromal cells and TAMs, by selectively up-regulating the expression of the chemokine receptor CXCR4 (Schioppa et al. 2003). Furthermore, it has been shown that HIF-1 activation mediates expression of the CXCR4 ligand CXCL12, a chemokine involved in angiogenesis and cancer metastasis (Ceradini et al. 2004; Muller et al. 2001). TAMs adapt to hypoxia by increased expression of HIF-inducible pro-angiogenic genes such as VEGF, βFGF, and CXCL8, as well as glycolytic enzymes (Nakao et al. 2005), matrix metalloproteinase-9 (MMP-9), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), COX-2, MMP-7, MMP-12 (Lewis and Pollard 2006).

At the tumor microenvironment, hypoxia affects accumulation and functions of myeloid cell populations other than TAMs, including the angiogenic monocytes expressing the tunica internal endothelial kinase 2 (Tie2) (De Palma et al. 2005) and the heterogeneous population of MDSCs (Gabrilovich et al. 2012). $Tie2^+$ monocytes are mainly clustered in hypoxic areas of solid tumors, in close proximity to nascent tumor vessels, where they are recruited by hypoxia-inducible chemotactic factors such as the CXCR4 ligand CXCL12 and Angiopoietin-2 (Ang-2) (Welford et al. 2011). Of note, ANG2 inhibition, by monoclonal antibodies, peptibodies, or CovX-Bodies, produced substantial antiangiogenic and antitumor responses in a variety of mouse tumor models (De Palma and Naldini 2011).

MDSCs isolated from murine tumors express high levels of metalloproteases, including MMP9 (Fang et al. 2009). MMP9 increases the bioavailability of VEGF sequestered in the extracellular matrix. Further, in the tumor microenvironment and in proangiogenic culture conditions, MDSCs acquire endothelial markers such as CD31 and VEGF receptor 2 (VEGFR2) and the ability to directly incorporate into tumor endothelium (Lin et al. 2009). In agreement, tumor refractoriness to anti-VEGF therapy was shown to be mediated by $CD11b^+GR1^+$ myeloid cells, able to provide tumor resistance to VEGF therapy via their release of Bv8 (prokineticin-1), a proangiogenic cytokine stimulated by their exposure to tumor cell-derived granulocyte colony-stimulating factor (G-CSF) (Shojaei et al. 2007). Moreover, hypoxia promotes the HIF-1α-mediated differentiation of MDSC in TAM, thus contributing to their intratumor accumulation (Herber et al. 2010). In addition, accumulation of neutrophils in tumor tissues is promoted by hypoxia-induced expression of CXCL8, as observed in bronchoalveolar carcinoma (Blengio et al. 2013), and by their increased survival observed in low oxygen conditions (Walmsley et al. 2011). In this regard, neutrophils accumulation in tumors is gaining relevance, as Fridlender and

colleagues recently proposed a new paradigm in which resident tumor-associated neutrophils (TANs) acquire a pro-tumor angiogenic phenotype, largely driven by TGFβ, to become "N2 polarized neutrophils" (Fridlender et al. 2009).

20.2.4 Immunosuppression

Myeloid cells are a major component of inflammatory reactions, and recent evidence has placed them at a prominent position in the regulation of tumor-associated immune suppression. In healthy individuals, myeloid progenitors differentiate in mature granulocytes, macrophages, or dendritic cells, whereas in pathological conditions, such as cancer, they expand into MDSCs. MDSCs have been observed in cancer, chronic infectious diseases, and autoimmunity. In tumor-bearing mice, MDSCs accumulate within primary and metastatic tumors, in the bone marrow, spleen, and peripheral blood. In cancer patients, MDSCs have been identified in the blood. MDSCs represent a heterogeneous population, comprising the monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (G-MDSCs) (Bosch et al. 2010), whose common characteristics are an immature state and the ability to suppress T-cell responses both in vitro and in vivo (Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg and Sinha 2009). MDSCs possess several mechanisms for immune suppression: (1) depletion of arginine, mediated by Arg1 and iNOS; (2) production of ROS; (3) post-translational modifications of T-cell receptor (TCR) mediated by peroxynitrite generation; (4) depletion of cysteine; (5) production of TGFβ; (6) induction of Tregs (Youn et al. 2008; Movahedi et al. 2008; Bronte et al. 2005; Nagaraj et al. 2007; Srivastava et al. 2010; Yang et al. 2006; Huang et al. 2006; Terabe et al. 2003; Jin et al. 2008; Bhardwaj et al. 2010).

Human MDSCs are generally identified as cells expressing the common myeloid marker CD33, lacking the expression of markers of mature myeloid and lymphoid cells, and able to suppress T-cell activation (Herber et al. 2010; Nagaraj and Gabrilovich 2010; Almand et al. 2001; Rodriguez et al. 2009; Zea et al. 2005; Vuk-Pavlovic et al. 2010; Liu et al. 2010; Filipazzi et al. 2007; Poschke et al. 2010; Hoechst et al. 2008). M-MDSC-mediated immune suppression does not require cell–cell contact, but utilizes up-regulation of iNOS and Arg1, as well as production of immunosuppressive cytokines (Gabrilovich and Nagaraj 2009; Corzo et al. 2009). On the contrary, G-MDSCs suppress antigen-specific responses using mechanisms, including the release of ROS, that require prolonged cell–cell contact between MDSC and T cell (Corzo et al. 2009).

A remarkable relation exists between MDSCs and TAMs. MDSCs are able to skew TAM differentiation toward a tumor-promoting type-2 phenotype (Sinha et al. 2007). The cross-talk between MDSCs and macrophages requires cell–cell contact, then MDSCs release IL-10 to reduce IL-12 production by macrophages. MDSCs from an IL-1β-enriched tumor microenvironment produce more IL-10 and are more potent down-regulators of macrophage-released IL-12 (Bunt et al. 2009). Because of their tumor-promoting activities, MDSCs are associated with type-2 immune

responses; however, accumulating evidence shows that MDSCs have characteristics of both M1 and M2 macrophages (Allavena et al. 2008).

In established tumors, available information suggests that TAM have a skewed M2-like phenotype (Fig. 20.1), which is characterized by an IL-12^{low}/IL-10^{high} phenotype. Autocrine IL-10 accounts, in part, for defective IL-12 production (Sica et al. 2000). In addition, TAMs release a variety of factors (IL-4, IL-6, IL-10, PGE_2, TGF-β1, and CSF-1) that have been shown to suppress the proliferation and cytotoxicity of T-cells and NK cells (Mantovani et al. 2002). Further, IL-10 and VEGF production by TAM prevents dendritic cell maturation, thus impairing efficient tumor-antigen presentation (Mantovani et al. 2002). TAMs produce a selected set of chemokines (CCL2, CCL22, CCL18). Among these, CCL18 produced by TAMs was identified as the most abundant chemokine in human ovarian ascites fluid (Schutyser et al. 2002). CCL18 is an attractant for naïve T-cells, thus promoting their accumulation in a peripheral microenvironment dominated by M2 cells, which might induce anergy. Interestingly, hypoxia enhances expression of the suppressive enzymes iNOS and arginase in TAM and MDSC (Melillo et al. 1995; Sica and Bronte 2007) (Fig. 20.2).

TANs might also contribute to immune suppression (Fridlender et al. 2009; Dumitru et al. 2012). Th is supported by recent studies which showed that depletion of neutrophils associated with more activated intratumoral $CD8^+$ T-cells and reduced tumor growth (Fridlender et al. 2009). To exert their immunosuppressive properties, neutrophils might degranulate and release arginase 1 pre-stored in their granules (Rotondo et al. 2009).

20.2.5 Iron Metabolism

Macrophages play an important role in iron homeostasis by recycling iron through phagocytosis of senescent red blood cells and rendering it available for processes like erythropoiesis. Mouse and human macrophage polarization is associated with differential regulation of iron metabolism (Cairo et al. 2011). IL-4-activated macrophages expressed high levels of CD163 and CD94 (heme uptake), low Ferritin (iron storage), and high Ferroportin (iron export). The high levels of intracellular heme pool together with induction of heme oxygenase result in production of CO, which has immunosuppressive activity. In contrast, IFNγ-activated macrophages show a CD163^{low}Ferritin^{high}Ferroportin^{low} phenotype (Cairo et al. 2011; Recalcati et al. 2010). Thus, M2-polarized macrophages are set in an iron-export mode that supports immunoregulation, promotion of matrix remodelling, and cell proliferation. Conversely, M1-polarized macrophages are set in an iron-retention mode that supports bacteriostatic and tumoristatic activity. Proteins like Hepcidin and human hemochromatosis protein (HFE) contribute to the iron homeostasis in macrophages by degrading ferroportin, which results in inhibition of iron release (Cairo et al. 2011). Thus, iron handling has emerged as a key property of macrophage polarized activation with broad implications in immunopathology.

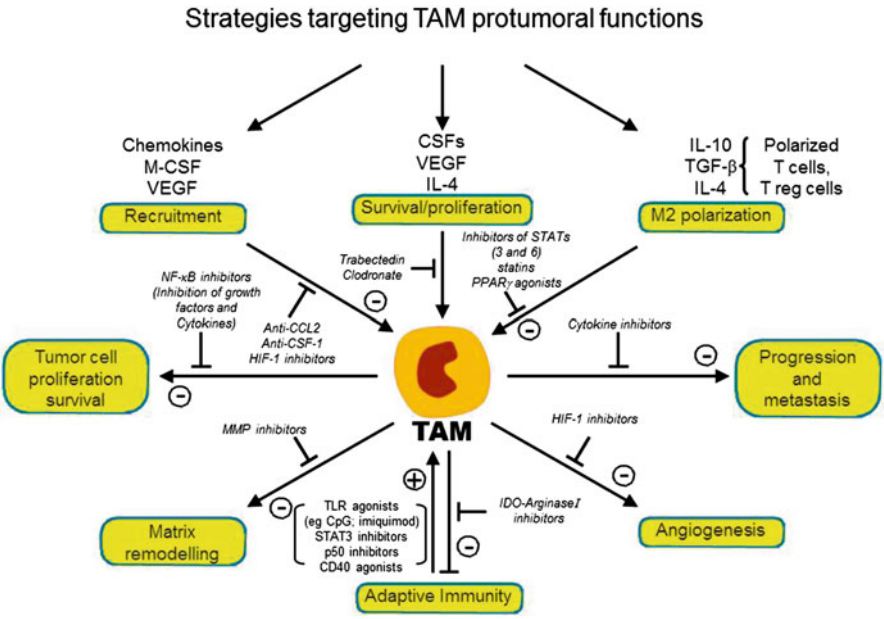

Fig. 20.2 Potential therapeutic approaches to prevent TAM pro-tumoral functions. TAM promote tumor progression by favoring angiogenesis, suppression of adaptive immunity, matrix remodelling, tumor progression, and metastasis. The figure summarizes strategies impairing selective TAM pro-tumoral functions (–) or restoring antitumor activities (+). Cytotoxic drugs (e.g., Yondelis) may decrease TAM number and prevent pro-tumoral functions. A similar result may be obtained by limiting TAM recruitment. Restoration of M1 immunity (STAT-3 and -6 inhibitors; anti-IL-10 plus CpG; CD40 agonists; IDO-iNOS-Arginase I inhibitors) would provide cytotoxic activity and re-activation of Th1 specific antitumor immunity. Inhibition of both pro-inflammatory cytokines and growth factors expression (NF-κB inhibitors) may disrupt inflammatory circuits supporting tumor growth and progression. MMPs inhibitors would prevent cancer cells spread and metastasis. Finally, inhibitors of TAM-mediated angiogenesis (HIF-1 inhibitors) would restrain blood supply and inhibit tumor growth. *M-CSF* macrophage-colony stimulating factor, *VEGF* vascular endothelial growth factor, *CSFs* colony-stimulating factors, *IL-* interleukin-, *TGF-β* transforming growth factor-β, *IDO* indoleamine 2,3-dioxygenase, *MMP inhibitors* matrix metalloproteinase inhibitors, *TLR agonists* toll-like receptor agonists, *STAT* signal transducer and activator of transcription, *NF-κB* nuclear factor-kappa B

20.3 Macrophage Targeting and Re-switch

Specific macrophage-targeted therapies are now emerging in the clinic (Sica and Mantovani 2012). In addition, therapeutic approaches not originally designed as macrophage oriented or specific have been found to affect macrophage activation and polarization.

Recruitment is a key determinant sustaining macrophage numbers at sites of inflammation and immunity. Monocyte attractants include members of the chemokine superfamily, CCL2/MCP-1 in particular, and growth factors interacting with

tyrosine kinase receptors, CSF-1 and VEGF (Qian et al. 2011; Wang et al. 1993; Barleon et al. 1996). CSF-1 receptor (c-fms) kinase inhibitors have been generated, and these molecules exhibit anti-angiogenic and anti-metastatic activity in acute myeloid leukemia and melanoma models (Ohno et al. 2006; Manthey et al. 2009). Chemokines and CSF-1 are more than monocyte attractants; they also promote M2-like skewing of macrophage function (Roca et al. 2009; Martinez et al. 2006; Zhang et al. 2010). Antibodies directed against CCL2/CCR2 have proven active in prostate and breast cancer (Qian et al. 2011; Loberg et al. 2007). A CCL2 inhibitor (bindarit) has proven active in preclinical models of cancer and vascular pathology, resulting in inhibition of monocyte recruitment (Gazzaniga et al. 2007); this agent is now undergoing evaluation for clinical use. Anti-CSF-1 antibodies and antisense oligonucleotides suppress macrophage infiltration and xenografts mammary tumor growth in mice (Aharinejad et al. 2002, 2004). VEGF inhibitors can decrease macrophage recruitment and this effect may contribute to their anti-angiogenic activity (Giraudo et al. 2004). In contrast, in response to neoadjuvant chemotherapy, altered composition in the immune microenvironment was found in breast cancer patients, with increased percentage of infiltrating myeloid cells (Ruffell et al. 2012).

Recent results suggest that in situ proliferation is a key determinant of macrophage accumulation during inflammation in the peritoneum and lungs (Jenkins et al. 2011; Davies et al. 2011). In the latter, IL-4 was found to sustain macrophage proliferation. If proliferation is indeed a major general mechanism sustaining macrophage accumulation, this may suggest novel approaches to reduce macrophage numbers in situ and a different perspective for anti-CSF-1/CSF-1R strategies. However, determining the actual significance of macrophage proliferation in humans in particular in T_H2-mediated inflammation is a major stumbling block in this perspective.

Reorienting and reshaping deranged macrophage polarization is a holy grail of macrophage therapeutic targeting (Mantovani and Sica 2010). Polarized phenotypes are reversible in vitro and in vivo (Sica et al. 2000; Hagemann et al. 2008; Duluc et al. 2009; Beatty et al. 2011). In proof of concept and in a large clinical study in ovarian cancer patients, IFNγ was found to activate TAM tumoricidal activity inducing a phenotype switch with unequivocal evidence of clinical responses (Allavena et al. 1990). In a model of pancreatic ductal adenocarcinoma, CD40 agonist antibodies promoted a remarkable antitumor effect and induced high expression of M1 markers (MHC class II and CD86) in macrophages (Beatty et al. 2011).

Modulation of macrophage function is an off-target effect for a number of diverse therapeutic agents. PPARγ agonists (Thiazolidinediones) have long been used in the treatment of diabetes. The evidence linking PPARγ to M2 polarization and hence to the homeostatic role of adipose-tissue macrophages (ATMs) shed fresh new light on their mode of action. Preclinical evidence suggests that PPARγ promotes M2-like polarization and homeostatic metabolic function in ATMs, and that alteration of this function is a key pathogenic feature in diabetes (Lu et al. 2011; Stienstra et al. 2008; Charo 2007). Other therapeutic strategies that have been reported to affect macrophage polarization include zoledronic acid (an agent used for preventing recurrence of breast cancer bone metastasis), statins (Fujita et al. 2010), trabectedin (Germano et al. 2010, 2013), and TLR ligands (e.g., imiquimod and CpG).

Overall, despite therapeutic macrophage targeting is in its infancy, accumulating evidence indicates that identification of mechanisms and molecules associated with macrophage plasticity and polarized activation provides a basis for macrophage-centered diagnostic and therapeutic strategies.

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Part V
Transcriptional and Epigenetic Mechanisms

Chapter 21
Role of NF-κB Activation in Macrophages

Toby Lawrence

Nuclear factor-κB (NF-κB) is the prototypical pro-inflammatory transcription factor, this is primarily based on its activation by pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), and pattern recognition receptors (PRRs) including Toll-like receptors (TLRs). In response to these pro-inflammatory signals NF-κB regulates the expression of other pro-inflammatory and cell survival genes that perpetuate the inflammatory response. It is this pivotal role in pro-inflammatory signaling pathways that has attracted a great deal of interest in NF-κB as a therapeutic target in inflammatory and autoimmune diseases. In light of their important role in orchestrating inflammation and immunity, NF-κB activation in macrophages has long been thought to be a major factor in the inflammatory response and consequently many inflammation-associated diseases. However, in recent years the development of sophisticated genetic tools to study the cell-specific role of the NF-κB in vivo has revealed previously unexpected anti-inflammatory and immunosuppressive roles for this pathway specifically in the macrophage lineage. In this chapter we will review these recent insights into the role of NF-κB activation in macrophages and related myeloid cells in the context of inflammation and immunity.

21.1 NF-κB Activation

Currently most of our knowledge of NF-κB signaling in inflammation has been gained from studying members of the IL-1 and TNF receptor families, and Toll-like microbial pattern recognition receptors (TLRs), which in fact belong to the

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IL-1R family (Karin and Ben-Neriah 2000; Takeuchi and Akira 2010). IL-1 and TNFα represent the archetypal pro-inflammatory cytokines that are rapidly released upon tissue injury or infection. TLRs recognize microbial molecular patterns, hence the term pattern recognition receptor (PRR), and represent a germline encoded non-self-recognition system that is hard-wired to trigger inflammation (Akira et al. 2006; Takeuchi and Akira 2010). However, there is some suggestion that endogenous ligands, or "danger" signals, may trigger TLRs during inflammation and tissue injury which may act to promote inflammation in the absence of infection (Karin et al. 2006). Although structurally different these receptors use similar signal transduction mechanisms to activate NF-κB which converge upon activation of IκB kinase (IKK) and the subsequent phosphorylation and degradation of IκB proteins, the endogenous inhibitors of NF-κB (Hayden and Ghosh 2008).

NF-κB is in fact a generic term for a dimeric transcription factor composed from a family of five subunits that share a REL homology domain and are therefore called REL proteins; REL (c-REL), RELA, RELB, p52, and p50 (Oeckinghaus and Ghosh 2009). The p52 and p50 subunits are sythesized as p100 (NFKB2) and p105 (NFKB1), respectively, that require proteolytic processing to p52/p50. There are at least two separate pathways for NF-κB activation in inflammation and immunity (Fig. 21.1), the "canonical" pathway is triggered by microbial products and pro-inflammatory cytokines such as TNFα and IL-1, as described above, this usually leads to activation of RELA or c-REL containing complexes (Karin and Ben-Neriah 2000), although recent studies suggest in some contexts the canonical pathway can regulate activation of RELB/p50 complexes (Shih et al. 2012). An "alternative" NF-κB pathway was described in 2001 (Senftleben et al. 2001), which is activated by TNF-family cytokines: lymphotoxin β (LTβ; TNFSF3), CD40 ligand (CD40L; TNFSF5), B cell activating factor (BAFF; TNFSF13B), and receptor activator of NF-κB ligand (RANKL; TNFSF11), but not TNFα, and results in activation of RELB/p52 complexes (Sun 2012). These pathways are characterized by the differential requirement for IKK subunits. The IKK complex consists of two kinase subunits; IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit IKKγ (NEMO). IKKβ regulates activation of the canonical pathway through phosphorylation of IκBs and requires the IKKγ subunit but not IKKα (Zandi et al. 1997; Ghosh and Karin 2002), whereas IKKα is required for activation of the alternative pathway through the phosphorylation and processing of p100, the precursor for p52 (Senftleben et al. 2001), but this is independent of both IKKβ and IKKγ (Ghosh and Karin 2002). NFKB1 (p100) has also been shown to act as an IκB for a small fraction of RelA-containing complexes, which can be mobilized in response to LTβ-mediated activation of IKKα (Basak et al. 2007) (Fig. 21.1). IL-1, TNFα or TLR-mediated NF-κB activity in macrophages is invariably associated with activation of the canonical pathway and RELA or c-REL. To our knowledge there is no evidence for activation of the alternative pathway, defined by IKKα-dependent p100 processing, in macrophages in response to these pro-inflammatory stimuli.

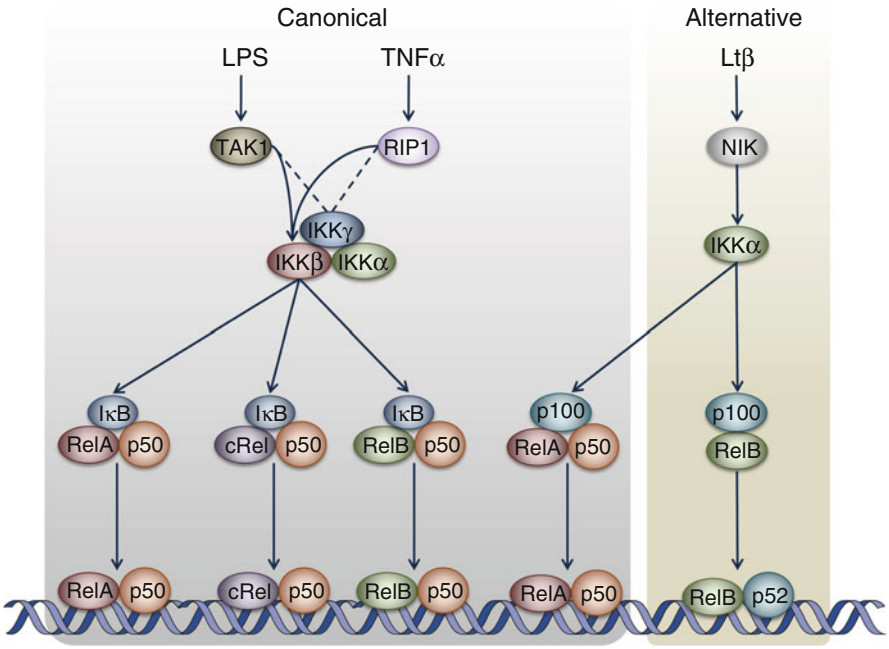

Fig. 21.1 The two pathways for NF-κB activation in inflammation and immunity: the "Canonical" pathway is triggered by microbial products, such as LPS and pro-inflammatory cytokines including TNFα. This pathway requires the IKKβ-mediated phosphorylation of IκB proteins, the endogenous inhibitors of NF-κB. IKKγ is a ubiquitin-binding protein that couples the IKK complex to upstream kinases such as TAK1 and RIP1. The canonical pathway usually leads to activation of RelA or cRel containing complexes, although recent studies suggest in some contexts the canonical pathway can regulate activation of RelB/p50 in response to LPS. The "alternative" NF-κB pathway is activated by TNF-family cytokines, such as lymphotoxin β (Ltβ), but not TNFα. Ltβ triggers NIK-dependent phosphorylation of IKKα and the subsequent phosphorylation and processing of p100 to p52, this results in activation of RelB/p52 complexes. A small fraction of RelA-complexes may also be bound by p100 and therefore IKKα-mediated p100 processing can lead to activation of "canonical" NF-κB in response to Ltβ. These pathways are characterized by the differential requirement for IKK subunits: IKKβ regulates activation of the canonical pathway through phosphorylation of IκBs and requires IKKγ but not IKKα, whereas IKKα is required for activation of the alternative pathway through the phosphorylation of p100, but this is independent of both IKKβ and IKKγ

21.2 Anti-inflammatory Roles for NF-κB Activation in Macrophages

RELA is the critical effector of the canonical pathway, as illustrated by the overlapping phenotypes of RelA and IKKβ knockout mice (Beg and Baltimore 1996; Li et al. 1999), but the embryonic lethality of RelA or IKKβ deficiency had hampered in vivo studies of inflammation. However, using fetal liver stem cells from

Rela$^{-/-}$ embryos to generate chimeric mice, Alcamo et al. (2001) showed that RelA expression in radiation-resistant tissue cells was required for the leukocyte recruitment in the lung after challenge with the bacterial product lipopolysaccharide (LPS), but mice that lack RelA expression in hematopoietic cells showed no defect in inflammation. This was quite surprising considering the strong activation of NF-κB in lung macrophages in response to LPS, and suggested a different role for NF-κB activation in macrophages. Using pharmacological inhibitors, we previously showed the involvement of NF-κB in both the onset and resolution of inflammation in a single model system (Lawrence et al. 2001), these studies confirmed the expected role of NF-κB in pro-inflammatory gene induction during the onset of inflammation, but also demonstrated a role for NF-κB in expression of anti-inflammatory genes and induction of leukocyte apoptosis during the resolution of inflammation. Inhibition of NF-κB in leukocytes, including macrophages, prevented apoptosis and prolonged the inflammatory response, which was in contrast to the generally accepted view that NF-κB was pro-inflammatory and anti-apoptotic in inflammatory cells. These early studies suggested a cell-specific role for NF-κB in inflammation and an anti-inflammatory role in recruited inflammatory cells such as macrophages.

Since the development of Cre/*lox*-mediated gene targeting (Sauer 1998) it has been possible to specifically target NF-κB pathway genes in different cell lineages, this approach has definitively demonstrated the anti-inflammatory roles of NF-κB activation in macrophages and other myeloid cells. One of the first such studies showed a protective role for NF-κB activation in macrophages during atherosclerosis (Kanters et al. 2003). Macrophages play a key role in the pathogenesis of atherosclerosis, which is considered an inflammatory disease (Weber et al. 2008). Kanters and colleagues tested the role of NF-κB activation in macrophages in a mouse model of atherosclerosis by generating myeloid-specific IKKβ knockout mice on an LDL-receptor deficient background (*Ldlr*$^{-/-}$) (Kanters et al. 2003). Deletion of IKKβ in myeloid cells increased the number and size of atherosclerotic lesions in *Ldlr*$^{-/-}$ mice on a high fat diet. There was the expected reduction in expression of pro-inflammatory cytokines by macrophages, including TNFα and IL-6, in IKKβ targeted mice but there was also a significant reduction in expression of the anti-inflammatory cytokine IL-10, illustrating that NF-κB activation in macrophages contributes to both pro- and anti-inflammatory cytokine production during atherosclerosis. Later, Greten et al. (2007) showed an anti-inflammatory role for IKKβ expression in myeloid cells in sepsis. Specific deletion of IKKβ in neutrophils and macrophages increased sensitivity of mice to endotoxin (LPS)-induced shock, associated with elevated plasma IL-1β as a result of increased pro-IL-1β processing in targeted cells. This was due to the inhibition of NF-κB mediated expression of the serine protease inhibitor PAI-2 in macrophages, which acts as an inhibitor of caspase-1 mediated IL-1β processing. In addition, Greten et al. confirmed a pro-apoptotic role for NF-κB in neutrophils which may also contribute to the anti-inflammatory role of NF-κB, as previously described (Lawrence et al. 2001). In fact, subsequent studies have shown that inhibition of NF-κB in myeloid cell progenitors inhibits apoptosis and results in the inappropriate expansion of neutrophils in the bone marrow (Hsu et al. 2011). In this study, it was shown that deletion of

IKKβ in myeloid progenitors could be protective in the context of infection, due to the increased mobilization of granulocytes from the bone marrow. In a parallel study it was shown that TNFα was a key mediator of myeloid progenitor cell apoptosis through activation of IKKβ (Mankan et al. 2011), however in this study the absence of IKKβ expression and expansion of myeloid cells resulted in increased IL-17 production and exacerbation of an experimental model of multiple sclerosis (EAE), demonstrating an immunosuppressive role for NF-κB activation in myeloid cells during autoimmune disease.

Our group has demonstrated the pro- and anti-inflammatory roles for IKKβ-mediated NF-κB activation during bacterial infection using lineage-specific gene targeting. In a model of Streptococcal pneumonia, when IKKβ was deleted in lung epithelial cells neutrophil recruitment and bacterial clearance was impaired, however in mice lacking IKKβ in myeloid cells neutrophil recruitment and bacterial clearance was enhanced (Fong et al. 2008). In addition, IKKβ deficient lung macrophages showed increased MHC II, NOS2, and IL-12 expression, which are hallmarks of "classical" or M1 macrophage activation (Gordon and Taylor 2005; Fong et al. 2008). IKKβ deletion in macrophages was shown to increase activation of STAT1 in response to the autocrine type I interferon (IFNαβ) or exogenous IFNγ (Fong et al. 2008). STAT1 is a critical mediator of M1 macrophage activation through regulation of genes such as CIITA, NOS2, and IL12B (Lawrence and Natoli 2011). Interestingly, expression of the IL-4 receptor α chain (CD124) was absent on IKKβ-deficient macrophages (Fong et al. 2008), suggesting these cells have lost the ability to respond to IL-4/IL-13 and develop an anti-inflammatory M2 phenotype (Martinez et al. 2009). These data suggest IKKβ is involved in the suppression of pro-inflammatory (M1) macrophages, and favors the development of anti-inflammatory M2 macrophages, this may have important implications for the role of NF-κB in tumor-associated macrophages (TAM), as discussed later in this chapter.

21.3 IKKα and Macrophage Activation

The "alternative" NF-κB pathway is characterized by the inducible phosphorylation of p100 by IKKα leading to activation of RELB/p52 heterodimers, the upstream kinase that activates IKKα in this pathway has been identified a NIK (NF-κB inducing kinase) (Senftleben et al. 2001). Genetic studies in mice have demonstrated the important role for this pathway in lymph organogenesis and B cell function (Bonizzi et al. 2004), however there are no reports of "bona fide" activation of the alternative NF-κB pathway in macrophages in response to pro-inflammatory stimuli, such as TLR ligands, TNFα, or IL-1β. This posed the question: What is the role of IKKα in the canonical IKK complex? We addressed this using transgenic mice that express a mutant form of IKKα, where two serine residues in the activation loop of the kinase had been mutated to alanine ($IKK\alpha^{AA}$) (Cao et al. 2001), therefore cells express a native IKK complex but lack the inducible activity of IKKα. Using macrophages from these mice, we showed IKKα

regulates the stability, and promoter recruitment, of RelA and c-Rel containing NF-κB complexes in the context of infection (Lawrence et al. 2005). This was mediated by the IKKα-dependent C-terminal phosphorylation and proteosomal degradation of RelA and c-Rel. Previous studies had shown that NF-κB has an important role in resistance to pathogen-induced apoptosis in macrophages (Park et al. 2002; Hsu et al. 2004). Our studies showed that IKKα had a pro-apoptotic function in macrophages exposed to pathogenic bacteria, through the inhibition of canonical NF-κB activation. Consequently, $IKK\alpha^{AA}$ mice were more resistant to infection, which was associated with increased inflammation due to enhanced survival of activated macrophages (Lawrence et al. 2005). Several subsequent studies have also shown that IKKα negatively regulates canonical NF-κB activation in various contexts (Li et al. 2005; Liu et al. 2007; Shembade et al. 2011). Exactly how IKKα activity affects the anti-inflammatory functions of IKKβ/NF-κB in macrophages is not clear. It may be that IKKα-mediated suppression of NF-κB regulated genes is promoter-specific, and primarily regulates the anti-apoptotic function of NF-κB in macrophages through the destabilization of RelA/c-Rel on specific anti-apoptotic gene promoters (Lawrence et al. 2005), and therefore prevents their persistent activation. However, other anti-inflammatory genes regulated by NF-κB, such as IL-10, may not be sensitive to this mechanism of inhibition. This would provide a mechanism for the parallel inhibition of pro-inflammatory signaling and cell survival in macrophages through the coordinated activation of IKKα and IKKβ (Fig. 21.2).

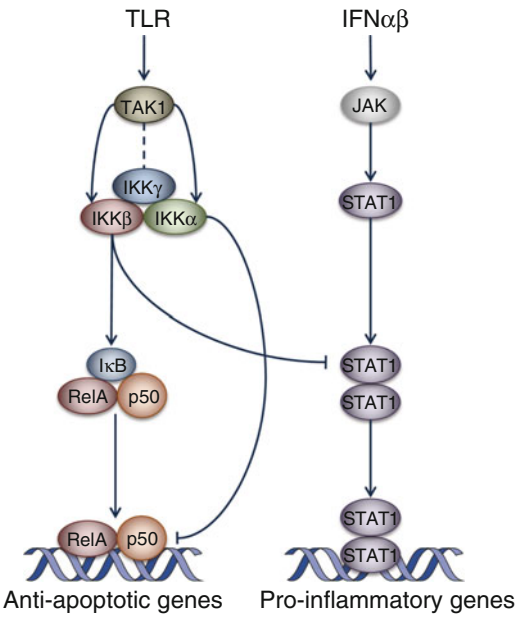

Fig. 21.2 Coordinated anti-inflammatory effects of IKKα and IKKβ in macrophages. IKKα promotes pathogen (TLR)-induced macrophage apoptosis through the inhibition of NF-κB-mediated transcription of anti-apoptotic genes. In parallel, IKKβ inhibits STAT1 activation in response to autocrine type I interferon (IFNαβ) signaling, inhibiting the expression of pro-inflammatory genes

21.4 IKKα and Antigen-Presenting Cell Function

Macrophages are professional antigen-presenting cells (APCs) (Hume 2008), although dogma dictates that dendritic cells (DC) have the unique ability to prime naïve T cells (Mellman and Steinman 2001), recent studies have shown that monocyte-derived DC, that may be considered a sub-population of macrophages (Hume et al. 2013), are critical for priming naïve T cells in the context of infection (Nakano et al. 2009; Cheong et al. 2010). Genetic studies have shown that several components of the alternative NF-κB pathway, including NIK, RelB, and p52, have an important role in DC function and T cell priming (Caamano et al. 1998; Franzoso et al. 1998; Wu et al. 1998; Weih et al. 2001; Speirs et al. 2004; Hofmann et al. 2011). Other studies have shown that LTβ-signaling is important to maintain homeostasis of specific DC subsets (Kabashima et al. 2005). Our own studies have established that IKKα activity is critical in CD11c-expressing cells, including macrophages and DC, for priming Th1 cell responses (Mancino et al. 2013). However, this function for IKKα was independent of NF-κB, in fact we showed that IKKα activation was required for interferon regulatory factor (IRF)-3 activity. Both IKKα and IRF-3 were required for Th1-cell priming by CSF2 (GM-CSF)-derived bone marrow macrophages. CSF2-derived macrophages have enhanced APC functions compared to their CSF1 (M-CSF)-derived counterparts and can be considered a model of M1-polarized macrophages (Krausgruber et al. 2011; Fleetwood et al. 2007). Our data showed that IKKα, but not IKKβ/NF-κB, is critical for the APC function of M1-like macrophages in the presence of CSF2, which may have important implications in Th1/Th17-driven inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and psoriasis (Hamilton and Tak 2009). Considering the immunosuppressive and anti-inflammatory roles of IKKβ in myeloid cells, IKKα may represent a much better target for the treatment of these autoimmune diseases.

21.5 NFKB1 and Macrophage Activation

Homodimers of the p50 subunit of NF-κB, which lacks a transactivation domain, have been shown to repress expression of NF-κB target genes and inhibit inflammation (Bohuslav et al. 1998), this is thought to be particularly important in cell autonomous LPS-induced tolerance in macrophages (Ziegler-Heitbrock 2001). Although increased p50 homodimer activation was reported to suppress TNFα production in LPS-tolerant macrophages, Gadjeva et al. (2004) have shown that p105/p50 deficient mice that are heterozygous for RelA (*Nfkb1*$^{-/-}$ *Rela*$^{+/-}$) show increased sensitivity to LPS-induced shock. These studies suggested anti-inflammatory roles for both p50 homodimer and RelA/p50 heterodimers in septic shock, which is in keeping with more recent studies targeting the canonical NF-κB pathway in sepsis through deletion of IKKβ specifically in myeloid cells (Greten et al. 2007; Fong et al. 2008).

Apart from sepsis, an anti-inflammatory role of NF-κB in macrophages has also been reported in inflammatory bowel disease (IBD), where *Nfkb1*$^{-/-}$ *Rela*$^{+/-}$ mice were more susceptible to *Helicobacter hepaticus* induced colitis (Erdman et al. 2001). Later studies have shown that administration of recombinant IL-10 reversed the exacerbation of *H. hepaticus* induced colitis, which was dependent on p105/p50 expression in macrophages (Tomczak et al. 2006a). These studies suggest NF-κB (p105/p50) can have anti-inflammatory roles by directly inhibiting expression of pro-inflammatory genes and by manipulating the expression or activity of anti-inflammatory cytokines such as IL-10. More recent studies have shown that p105/p50 is required for the M2-polarization of macrophages in the context of parasitic infection (Porta et al. 2009). In these studies, they suggest p50 homodimers inhibit interferon (IFN) β expression in LPS-tolerant and M2-polarized macrophages therefore blocking the autocrine activation of STAT1, a critical mediator for M1 macrophage activation. However, it is not clear from these studies that the M1-polarization of *Nfkb1*$^{-/-}$ macrophages is due to a loss of p50-homodimer-mediated repression of IFNβ. The gene product of *Nfkb1* (p105) is also a regulator of the MAP kinase TPL2 (Gantke et al. 2012), which has been shown to be required for ERK-mediated inhibition of IFNβ expression in macrophages (Kaiser et al. 2009). In *Nfkb1*$^{-/-}$ macrophages, TLP2 is destabilized preventing LPS-induced ERK activation and therefore increasing expression of IFNβ (Tomczak et al. 2006b; Yang et al. 2011), which suggests p105-mediated TPL2 regulation is an additional mechanism for the increased M1-activation phenotype in p105/p50 deficient macrophages. TPL2-mediated ERK activation is also associated with increased c-Fos and IL-10 expression in macrophages (Tomczak et al. 2006b; Kaiser et al. 2009), c-Fos has also been shown to inhibit NF-κB-mediated pro-inflammatory gene expression in macrophages (Koga et al. 2009). Therefore, the deletion of p105/p50 in macrophages inhibits several mechanisms for feedback control of pro-inflammatory (M1) macrophage activation, through prevention of both p50:p50-mediated gene repression and TPL2-dependent ERK activation. Interestingly, IKKβ-mediated phosphorylation of p105 has also been shown to be required for TPL2 activation (Gantke et al. 2012), this may provide an additional anti-inflammatory mechanism for IKKβ in macrophages through TPL2-ERK-dependent activation of c-Fos and IL-10 expression (Fig. 21.3).

21.6 NF-κB in Tumor-Associated Macrophages

Inflammation is a hallmark of cancer, and several studies in mouse models of colitis-associated colorectal cancer (CAC) and hepatocellular carcinoma (HCC) suggest a tumor-promoting role for NF-κB activation in TAM (Greten et al. 2004; Maeda et al. 2005). The targeted deletion of IKKβ in myeloid cells resulted in a marked reduction in tumor burden in a model of chemically (DEN)-induced HCC (Maeda et al. 2005). It was also shown in another genetic model of HCC that inhibition of NF-κB activation through over-expression of the endogenous inhibitory

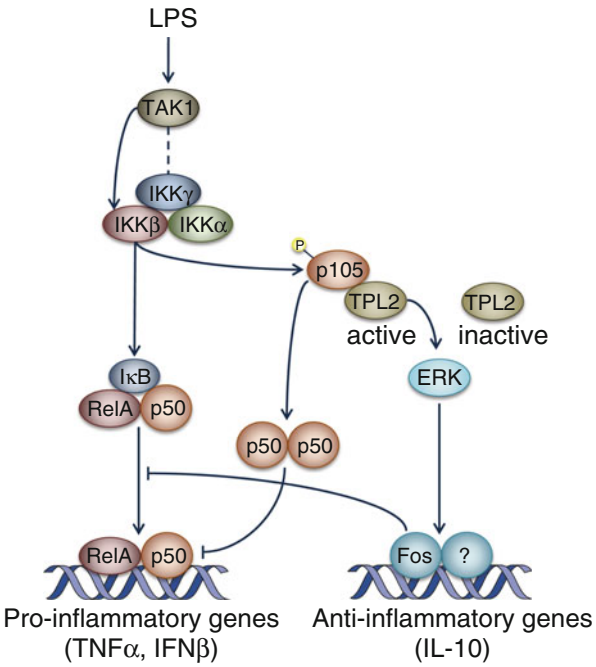

Fig. 21.3 Multiple mechanisms for NFKB1 (p105/p50)-mediated regulation of macrophage activation. Homodimers of p50 have been shown to repress transcription of pro-inflammatory genes such as TNFα and IFNβ through competition with RelA:p50 heterodimers. The kinase TPL2 is stabilized by p105 in its active form, IKKβ-mediated phosphorylation of p105 in response to TLR signaling (LPS) results in TPL2-mediated activation of ERK and Fos. In the absence of p105, TPL2 is inactive inhibiting activation of ERK. Fos activation is linked with positive regulation of IL-10 expression and inhibition of NF-κB-regulated pro-inflammatory genes, including TNFα and IFNβ

protein IκBα reduced tumor formation by blocking the tumor-promoting function for TNFα (Pikarsky et al. 2004). Naugler and colleagues demonstrated the TLR adaptor protein MyD88 was required upstream of NF-κB activation in DEN-HCC for IL-6 expression by TAM, and IL-6 production was linked to tumor growth through activation of STAT3 (Naugler et al. 2007). These studies also demonstrated that hepatocyte necrosis during tumor development stimulated pro-inflammatory cytokine production by liver macrophages (Kupffer cells) through the release of endogenous TLR ligands (Lawrence et al. 2007). Other studies have also shown that hepatocyte apoptosis during HCC was linked to increased TNFα (Maeda et al. 2005) and IL-6 production by Kupffer cells (Luedde et al. 2007), which promoted tumor growth through compensatory proliferation of hepatocytes. In this context, the release of endogenous "danger signals" and macrophage activation during carcinogenesis was linked with tumor promotion (Karin et al. 2006), however the release of such signals during chemotherapy or radiotherapy of established tumors

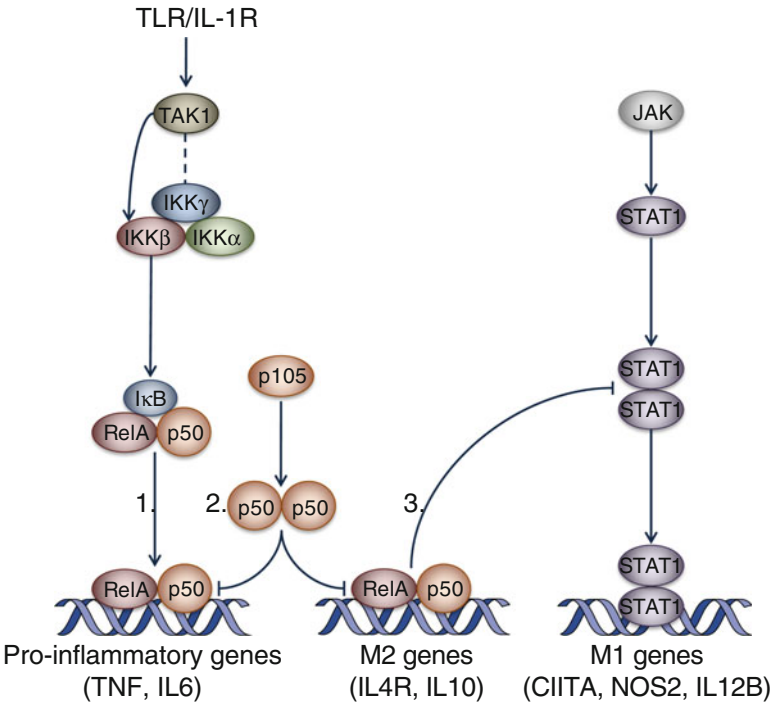

Fig. 21.4 NF-κB-mediated regulation of tumor-associated macrophage (TAM) polarization. IKKβ/NF-κB activation in TAM has been associated with both pro- and anti-inflammatory activity. (*1*). Products from necrotic tumor cells have been shown to trigger pro-inflammatory cytokine production, including TNFα and IL-6, through activation of IKKβ/NF-κB. (*2*). In other studies, p50 overexpression in TAM has been suggested to inhibit pro-inflammatory activation and promote an M2-like phenotype through inhibition of NF-κB activation (RelA:p50). (*3*). IKKβ/NF-κB activation has also been shown to inhibit M1-macrophage activation and promote the M2-like phenotype of TAM

may in fact be beneficial through the "re-activation" of TAM and triggering anti-tumor immune responses (Apetoh et al. 2007; Hagemann et al. 2007; Fig. 21.4).

IKKβ deletion in myeloid cells has also been shown to reduce growth of tumors in CAC (Greten et al. 2004). However, initiation of carcinogenesis in colonic epithelial cells was not affected, despite reduced tumor growth, indicating that NF-κB activation in myeloid cells has a role in tumor promotion and not initiation in this model (Greten et al. 2004). More recently, NF-κB activation in myeloid cells was also shown to contribute to the acceleration of lung carcinogenesis by tobacco smoke (Takahashi et al. 2010), again in these studies NF-κB activation in lung macrophages was shown to increase production of pro-inflammatory cytokines including TNFα and IL-6 that were linked to tumor-promotion.

21.7 Is NF-κB Pro- or Anti-inflammatory in TAM?

The studies described above in mouse models of HCC, CAC, and lung carcinogenesis suggest NF-κB activation in TAM has a pro-inflammatory role through the production of cytokines such as TNFα and IL-6, that would reflect an M1-like macrophage phenotype, this is in contrast to the anti-inflammatory M2-like phenotype of TAM described in other mouse models and human cancers (Biswas et al. 2006; Sica et al. 2008). Although NF-κB is primarily considered to be a major pro-inflammatory transcription factor, as described above NF-κB activation specifically in macrophages can also regulate anti-inflammatory pathways (Lawrence and Fong 2010). These anti-inflammatory functions for NF-κB in macrophages somehow conflict with the proposed pro-inflammatory role for NF-κB in TAM. It is clear that NF-κB activation in TAM has a role in tumor promotion (Hagemann et al. 2009), but is this due to pro- or anti-inflammatory roles for NF-κB in macrophages? For example, defective NF-κB function has been demonstrated in TAM from chemically induced fibrosarcomas associated with an M2-like phenotype (Biswas et al. 2006; Saccani et al. 2006). This defect was attributed to the over-expression of nuclear p50 that can act as a dominant-negative inhibitor of pro-inflammatory NF-κB-dependent genes, including TNFα (Ziegler-Heitbrock 2001). TAM from p105/p50 deficient mice re-gained a pro-inflammatory (M1) phenotype associated with reduced tumor growth; thought to be due to restored canonical NF-κB activity. However, as discussed above, recent studies have also shown that p105/p50 can actively promote the M2-polarization of macrophages independently of pro-inflammatory cytokines (Porta et al. 2009), that may contribute to tumor growth. NF-κB activation has also been associated with tumor regression in a mouse mammary carcinoma model (Guiducci et al. 2005), the TLR9 agonist CpG was used to activate NF-κB in combination with an IL-10 receptor blocking antibody and the chemokine CCL16 to promote T cell infiltration and activation, TAM were redirected towards anti-tumor functions by CpG with the acquisition of an M1 phenotype. In contrast, we have shown that blocking NF-κB activation in TAM isolated from established ovarian cancers increased their tumoricidal activity and switched their tumor-promoting M2-like phenotype towards M1 activation (Hagemann et al. 2008). These studies suggest that NF-κB activation maintains the M2-like phenotype of TAM in this model. Targeted deletion of IKKβ in TAM was also associated with increased activation of STAT1 and expression of IL-12, NOS2, and MHC II, hallmarks of M1 macrophage activation (Hagemann et al. 2008). These studies conflict with data from spontaneous HCC and CAC models described above (Greten et al. 2004; Pikarsky et al. 2004; Maeda et al. 2005; Luedde et al. 2007; Naugler et al. 2007), however in these experiments TAM isolated from tumors in advanced stages and it is possible the role of NF-κB activation in TAM may differ depending on the stage of tumor development and the tumor microenvironment. One could envisage that pro-inflammatory (M1-like) macrophage activation would be associated with necrosis and the breakdown of epithelial barrier function during the initiation of carcinogenesis in mouse models of HCC and CAC, respectively. However,

in proliferative and vascularized areas of solid tumors M2-like macrophages may dominate (Lewis and Pollard 2006; Sica et al. 2008), NF-κB activation may have differential roles in these distinct populations of TAM (Hagemann et al. 2009). The balance between these different macrophage phenotypes can clearly impact tumor progression and the molecular mechanisms involved may represent important therapeutic targets.

21.8 Future Perspectives

Despite being known as the prototypical pro-inflammatory transcription factor, it is clear that NF-κB can have important anti-inflammatory roles in macrophages. The consequences of these "non-canonical" functions for NF-κB are only beginning to be unraveled. There are clear indications in the context of infection that NF-κB activation in macrophages and other myeloid cells may well be to the advantage of the pathogen, the evolution of virulence factors that specifically trigger this "anti-inflammatory" mode of NF-κB activation has yet to be explored. There is strong evidence that NF-κB activation is critical for the tumor-promoting functions of macrophages. However, this may be attributed to both pro- and anti-inflammatory roles for NF-κB. The specific role of NF-κB in sub-populations of macrophages remains to be determined, this may require more robust markers and the generation of new transgenic mice that allow the targeting of NF-κB activation in macrophage subsets. The characterization of the molecular mechanisms that control the specificity of the NF-κB response in macrophages will be key to unraveling this complexity.

Currently one has to be pessimistic about targeting the NF-κB pathway per se in the treatment of inflammatory diseases based on these important anti-inflammatory functions in macrophages and other myeloid cells. However, with a better understanding of the mechanisms that control the specificity of the NF-κB response we may be able to identify new molecular targets in this pathway that could be beneficial in the treatment of chronic inflammatory diseases. On the other hand, the prospects of targeting NF-κB in the treatment of cancer look much more promising, in this case both the pro- and anti-inflammatory functions of NF-κB in the myeloid lineage are undesirable. Furthermore, there is increasing evidence that NF-κB activation in malignant cells is also an important contributing factor in carcinogenesis.

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Chapter 22
Interferon Regulatory Factors: Role in Transcriptional Regulation of Macrophage Plasticity and Activation

H.L. Eames and I.A. Udalova

The interferon regulatory factor (IRF) family of transcription factors was originally implicated in anti-viral responses and Type I interferon (IFN) production. Subsequent studies revealed their multifaceted role in regulation of anti-microbial responses and cell differentiation. In particular, IRF8 is now known as one of the major factors regulating myeloid cell growth and differentiation, while IRF5 and IRF4 are associated with M1 and M2 macrophage polarisation, respectively. In addition, the IRF transcription factors appear to provide a mechanism for conferring signal specificity to expression of immune genes regulated by NFκB. For example, IRF3, whose activation by upstream kinases occurs in response to TLR4 stimulation by lipopolysaccharide (LPS) and TLR3 stimulation by double stranded RNA (dsRNA), is not activated in many other NFκB inducing signalling pathways. Another emerging feature of the IRF family is that different members of the family seem to target specific gene subsets, with IRF3 being essential for anti-viral Type I IFN responses, and IRF5 playing a key role in induction of pro-inflammatory cytokines and chemokines. This chapter will discuss the multifaceted functions of IRF proteins in macrophage biology.

22.1 The IRF Family of Transcription Factors

The IRF family of transcription factors consists of nine family members in mammals: IRF1, IRF2, IRF3, IRF4 (also known as PIP, LSIRF or ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP) and IRF9 (also known as ISGF3γ)

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(Tamura et al. 2008). The IRFs were first characterised as transcriptional regulators of Type I IFN and IFN-induced genes, but it is becoming clear that they play a pivotal role in both the regulation and development of host defence beyond the IFN system, and in particular in macrophages. The members of the IRF family that have been shown to have important roles in macrophage biology include IRF1, IRF2, IRF3, IRF4, IRF5, IRF7 and IRF8, and will be discussed in this chapter. In addition to macrophages, IRF1/IRF2 play important roles in NK cells, IRF6 is expressed in keratinocytes, IRF3/IRF7 regulate Type I IFN production by pDCs and fibroblasts, IRF1/IRF2/IRF4 are active in T-cells, and IRF4/IRF5/IRF8 have roles in B-cells. In addition to their roles in immune regulation, the IRFs are also involved in regulation of the cell cycle, apoptosis and tumour suppression (Honda et al. 2006).

Structurally, the IRFs comprise a 120 amino acid DNA-binding domain (DBD) at their N-terminus, which consists of five well-conserved tryptophan repeats and shares structural homology with the Myb family of oncoproteins (Veals et al. 1992). The IRF DBD forms a helix-turn-helix motif and recognises a DNA sequence termed the Interferon-Stimulated Response Element (ISRE, $^{A}/_{G}$NGAAANNGAAACT) (Darnell et al. 1994) of which 5′-GAAA-3′ is described as the core binding motif for the domain, as determined by analysis of the crystal structure of IRF1 in complex with DNA (Escalante et al. 1998). Virally encoded forms of the IRFs (vIRFs), such as those identified in the genomes of human herpes virus 8 (HHV8) and Kaposi sarcoma herpes virus (KSHV) (Moore et al. 1996; Russo et al. 1996), lack several of the tryptophan residues in the DBD, so cannot bind to DNA and act as dominant negative mutants of IRF proteins (Ning et al. 2011). This highlights the importance of the IRFs in antiviral responses if viruses are trying to overcome IRF activity in particular in order to propagate.

At the C-terminus, most of the IRFs (except IRF1, IRF2 and IRF6) possess an IRF Association Domain (IAD), which is important for homo- and hetero-dimer formation between IRF family members upon activation, and is structurally similar to the Mad-Homology 2 (MH2) domain of the Smad family of transcription factors (Qin et al. 2003; Takahasi et al. 2003). IRF1 and IRF2 possess a variation of the IAD domain, known as IAD2, which displays similarity to the PEST domain of PU.1. The IAD is also an important interface for protein–protein interactions with unrelated proteins, such as PU.1 and STAT proteins, which can further define the nucleotide sequences adjacent to the core IRF-binding motif to which IRF protein complexes can bind.

IRF transcriptional activities are varied, resulting in either activation and/or repression, often depending on the protein complexes formed with co-factors. It can be said that the unique function of an IRF is determined by many elements, including: cell-type and stage-specific expression, ability to activate or repress transcription of target genes, post-translational modifications, and ability to interact with other IRFs and transcriptional activators/repressors.

22.2 IRF1 and IRF2: Positive and Negative Regulators of Gene Expression

IRF1 and IRF2 were the first members of the IRF family to be described in 1988, and their discovery led to the search for more members of the IRF family, in particular those that respond directly to viral infection (IRF3/IRF7).

22.2.1 IRF1 Positively Regulates Gene Expression in Macrophages

IRF1 is expressed at low resting levels in many cell types, including T-cells, NK cells and macrophages, and is inducible by both IFNα and IFNβ, which highlights the role of IRF1 in Type I IFN responses (Harada et al. 1989). IRF1 can bind to the ISRE element of the enhanceosome structure at the *Ifnb1* promoter (Escalante et al. 1998) (see section on IRF3/IRF7); however cells from *Irf1*$^{-/-}$ mice are not impaired in IFNα or IFNβ production following viral infection (Matsuyama et al. 1993), suggesting that IRF1 may be involved in later phases of Type I IFN expression, possibly as part of an autocrine positive-feedback loop, as has been described for IRF7 in fibroblasts (Marié et al. 1998).

IRF1 can also positively regulate expression of the genes encoding IL-12p40 and IL-12p35 in macrophages; therefore *Irf1*$^{-/-}$ mice exhibit a severe defect in the production of IL-12, an essential cytokine for T_H1 differentiation (Taki et al. 1997). As a result, *Irf1*$^{-/-}$ mice exhibit a bias towards T_H2-type immune responses and are unable to provide protection against *Leishmania major* infection (Lohoff et al. 1997). A lack of NK cells in *Irf1*$^{-/-}$ mice (Ogasawara et al. 1998) may also be partially responsible for the lack of IL-12 production, as NK cells produce IFNγ, which stimulates macrophages to produce IL-12. The iNOS gene (*Nos2*) also fails to be induced in *Irf1*$^{-/-}$ macrophages, which means reduced levels of nitric oxide catalysis, which is key for the effector phase of T_H1 responses (Kamijo et al. 1994).

IRF1 can interact directly with MyD88 (Negishi et al. 2006), and TLR9 engagement leads to post-translational modifications that further activate IRF1 in a process known as "IRF1-licensing", which leads to more efficient migration into the nucleus than non-MyD88 associated IRF1. *Irf1*$^{-/-}$ macrophages stimulated with IFNγ plus CpG are therefore impaired in their production of IFNβ, iNOS and IL-12p35 (Negishi et al. 2006). Hence, IRF1 is critical for IFNγ enhancement of the TLR-dependent gene induction programme.

22.2.2 IRF2 Can Both Antagonise and Co-operate with IRF1 Activity in Macrophages

IRF2 is constitutively expressed in many cell types including B-cells, T-cells and macrophages, and is upregulated by direct binding of IRF1 or IRF9 to the *Irf2* promoter, and by IFNβ (although to a lesser extent than IRF1) (Harada et al. 1994). Despite the lower inducibility of IRF2, an unstimulated cell normally contains more IRF2 than IRF1 protein due to higher stability (8 h versus 30 min half-life, respectively (Harada et al. 1993). Unlike IRF1, IRF2 contains a repression domain (aa325–349), and can negatively regulate Type I IFN gene expression by competing with IRF1 for promoter binding sites. IRF2 is therefore often considered an antagonist of IRF1 activity, and is important for inhibiting expression of target genes. For example, IRF1 positively regulates caspase-1 (*Casp1*) gene expression, whereas IRF2 negatively regulates the same gene ($Irf2^{-/-}$ macrophages show enhanced expression of caspase-1, which may explain accelerated apoptosis upon IFNγ and LPS stimulation) (Cuesta et al. 2007). It is thought that IRF2 acts as an inhibitor by acting as a substrate for histone acetyltransferase (HAT) enzymes such as PCAF and p300 (Masumi and Ozato 2001) to inhibit core histone acetylation and promotion of an active euchromatin environment for transcription. Similarly, vIRF1 can interact with p300 to inhibit core histone acetylation, which results in repressed transcription of IFN-responsive genes (Burysek et al. 1999).

Like IRF1, IRF2 is also important for T_H1 differentiation, as $Irf2^{-/-}$ mice show impaired production of IL-12 by macrophages (Salkowski et al. 1999). Interestingly, IRF2 may therefore be co-operating with IRF1 to positively regulate IL-12 gene expression, rather than acting as a transcriptional repressor, as described above. Alternatively, a lack of NK cells in $Irf2^{-/-}$ mice (Lohoff et al. 2000), like in the $Irf1^{-/-}$ mice, may also contribute to lower levels of IL-12 production by macrophages, via a lack of IFNγ production. On the other hand, the expansion of basophils in $Irf2^{-/-}$ mice, which results in increased production of IL-4, could be polarising macrophages to an M2 ($IL\text{-}12^{low}$) phenotype and T-cells toward a T_H2 phenotype (Hida et al. 2005). In relative terms, IRF2 contributes less to IL-12 production than IRF1, and a direct effect of IRF2 at the *IL12b* promoter has not been detected; so it is likely that IRF2 mediates positive gene induction by interactions with IRF1 or IRF8 via its IAD2 domain.

22.3 IRF3 and IRF7: Regulation of Type I IFN Responses

IRF3 and IRF7 are important transcription factors that together with NFκB and AP-1 regulate Type I IFN gene expression in macrophages, a process that is essential for host antiviral responses.

22.3.1 Activation of IRF3 and IRF7 in Macrophages

IRF3 is a constitutively expressed protein, which is not itself induced by Type I IFN or viral signalling (Hiscott et al. 1999). Instead, IRF3 resides in the cytoplasm in its latent, inactive conformation, in which two autoinhibitory domains that flank the IRF3 IAD [aa9–240 and aa380–427 (Lin et al. 1999)] are condensed so that the N-terminus and C-terminus of IRF3 interact, thus masking the DBD and active hydrophobic surface of the IAD (Qin et al. 2003). Upon viral infection, phosphorylation of C-terminal residues of IRF3 occurs, leading to re-organisation of these autoinhibitory elements and unfolding of the IRF3 latent structure. Consequently, this mediates realignment of the DBD and increased accessibility of the IAD interface, which enables homo- or hetero-dimerisation of IRF3 and interactions with co-activator molecules such as CBP/p300, both of which are essential for IRF3 transcriptional activity. Studies in vitro and in fibroblasts have identified that the crucial serine residues for alleviation of IRF3 autoinhibition are located in two C-terminal clusters: "Site 1" (Ser385-Ser386) and "Site 2" (Ser396–Ser405) (Panne et al. 2007a; Mori et al. 2004). It has since been determined that IRF5 and IRF7 also possess autoinhibitory domains (aa455–504 and aa238–410, respectively); therefore, post-translational phosphorylation events are also crucial for IRF5 and IRF7 activation (Barnes et al. 2002; Marié et al. 2000).

IRF7 is highly homologous to IRF3, yet unlike IRF3 its expression is strongly induced by Type I IFN signalling. IRF7 is expressed to high levels in plasmacytoid dendritic cells (pDCs), and low basal levels are present in many other cell types including macrophages (Ning et al. 2011). Latent IRF7 molecules reside in the cytoplasm in an autoinhibitory conformation, similar to IRF3. However the majority of IRF7 molecules are generated during the onset of viral infection, in particular by interferon-stimulated gene factor 3 (ISGF3), a heterotrimeric transcriptional activator complex consisting of IRF9 (induced by IFNγ), STAT1 and STAT2, which is triggered upon binding of Type I IFNs to their receptor and goes on to bind to the *Irf7* promoter (Sato et al. 1998). Newly synthesised and basal IRF7 molecules are activated by phosphorylation to induce dimerisation and nuclear translocation, and the region Ser471–Ser487 contains the critical phosphorylation sites (Lin et al. 2000), as mutation of these residues results in abrogation of IRF7s ability to transactivate Type I IFN promoters in fibroblasts (Marié et al. 2000).

22.3.2 IRF3/IRF7 Are Integral to the IFNβ Enhanceosome

Macrophages defective in the expression of both IRF3 and IRF7 completely fail to induce Type I IFN responses, and only reconstitution of both of these proteins together results in a normal anti-viral response, demonstrating that IRF3 and IRF7 have essential and distinct roles in Type I IFN gene expression (Sato et al. 2000). IRF3 and IRF7 can form homo- or hetero-dimers with each other once activated, and

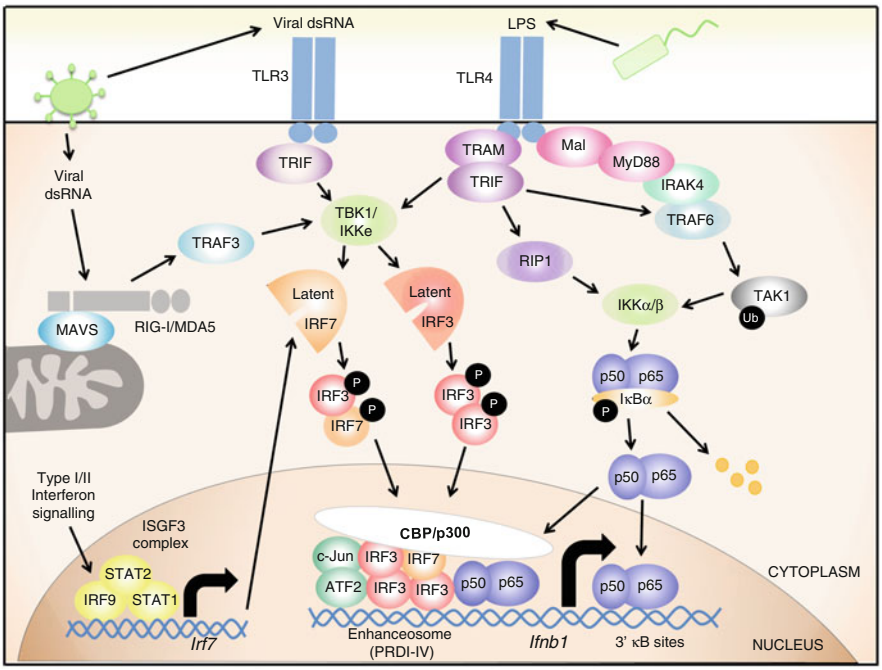

Fig. 22.1 Induction of Type I Interferon in response to viral and bacterial stimulation in myeloid cells. Viral dsRNA stimulates both transmembrane TLR3 and intracellular RIG-I/MDA5 receptors, resulting in the activation of the protein kinases TBK1 and IKKε. These kinases are also activated by LPS stimulation of TLR4, via the TRIF adaptor protein (also the case for IRF3). Phosphorylation of latent IRF7 (induced by the ISGF3 complex upon Type I/II interferon signalling) and latent IRF3 by TBK1/IKKε leads to homo/hetero-dimerisation of activated IRF3 and IRF7, which translocate to the nucleus to form part of the IFNβ enhanceosome structure at the *Ifnb1* promoter, along with AP-1 (c-Jun/ATF2) and NFκB (p65/p50). NFκB is activated by both the MyD88-dependent and MyD88-independent (TRIF) pathways upon LPS stimulation of TLR4, leading to phosphorylation and degradation of the NFκB inhibitor (IκBα) by IKK kinases, which allows NFκB to translocate to the nucleus. NFκB can form part of the enhanceosome structure at the *Ifnb1* locus, as well as binding to downstream κB sites, all of which are crucial for maximal IFNβ production by myeloid cells

in particular have been shown to co-operate in IFNβ induction, as part of the enhanceosome structure that assembles in a 60 bp virus-inducible region of the *Ifnb1* promoter (Merika and Thanos 2001) (see Fig. 22.1). The enhanceosome consists of two IRF dimers (either IRF3 or IRF7 homo- or hetero-dimers), one NFκB dimer (p65/p50) and one AP1 complex (ATF2/c-Jun) that independently bind to their respective binding sites, which are clustered together and described as the four positive regulatory domains (PRD-IV) of *Ifnb1*. These transcription factor complexes co-operatively interact with one another, to form the complete enhanceosome structure, as shown by the crystal structure of the complex bound to DNA (Merika and Thanos 2001; Panne et al. 2007b). This leads to the recruitment of histone acetyl transferase enzymes (HATs) and other chromatin remodelling activities that are

necessary for the initiation of *Ifnb1* gene expression (Agalioti et al. 2000). It is thought that the enhanceosome complex is more stable and efficient at inducing transcription than any of the individual components bound independently to the promoter, therefore the enhanceosome is optimal for robust IFNβ production in response to viral infection, especially in the situation of limiting concentration of key transcription factors (Apostolou and Thanos 2008). Alternative models of transcriptional regulation of the *Ifnb1* gene have also been discussed: for example, in LPS-stimulated MDDCs (monocyte-derived dendritic cells) and macrophages, which are characterised by high levels of NFκB, the contribution of multiple functional κB (and possibly IRF) sites outside of the enhanceosome region is essential for maximal level of gene induction (Goh et al. 2010) (see Fig. 22.1). This is consistent with data that human macrophages rapidly produce IFNβ mRNA upon LPS stimulation in a response that subsides after 8 h and is dependent on both IRF3 and NFκB (Reimer et al. 2008). Genome-wide analysis of gene expression in primary mouse B-cells and macrophages demonstrated that LPS induces a subset of "interferon regulated" genes, including *Ifnb1*, *Cxcl10*, *Ccl5*, *Isg15* and *Ifit1* (Ogawa et al. 2005; Doyle et al. 2002). Moreover, the ISRE was identified as the most highly significant motif enriched in the promoter sequences of LPS target genes, many of which dependent on IRF3 (Ogawa et al. 2005). LPS-induced IRF3 is recruited to promoters of a subset of tightly regulated primary response genes, where it instigates nucleosome remodelling and promotes gene expression (Ramirez-Carrozzi et al. 2009), thus portraying a role for IRF3 in macrophages away from viral activation.

22.3.3 Activation of IRF3 and IRF7 via the Protein Kinases TBK1/IKKε

TBK1 (TANK-binding kinase I) and IKKε (Inhibitor of NFκB kinase ε) have been identified as two protein kinases with the ability to directly phosphorylate IRF3 and IRF7 upon viral activation, which is sufficient to facilitate their nuclear translocation and DNA binding (Fitzgerald et al. 2003). As activation of NFκB is normal in *Tbk1*$^{-/-}$ and *Ikki*$^{-/-}$ cells, this demonstrates the specificity of these kinases in activation IRF3 and IRF7 and not NFκB. Studies in fibroblasts suggest that TBK1 is principally involved in IRF3 and IRF7 activation, whereas IKKε has a more accessory role, in which it regulates only a subset of interferon-responsive antiviral genes (Tenoever et al. 2007). This is highlighted by the fact that reconstitution of *Tbk1*$^{-/-}$ cells with IKKε only partially compensates for the lack of TBK1 in terms of IRF3-induced *Ifnb1* expression, which demonstrates only a partial redundancy between the two kinases (Perry et al. 2004), however this may be a cell type-specific effect.

IRF3/IRF7 can be phosphorylated by TBK1/IKKε by either one of the two pathways: the TLR-dependent pathway, or the TLR-independent cytosolic pathway, both of which converge at the point of TBK1 activation. Both IKKε and TBK1 associate with the PRR adaptor protein TRIF (TIR domain-containing adaptor-inducing IFNβ), and these interactions are facilitated by NAP1 (NAK-associated protein 1)

(Sasai et al. 2005). IRF3 and IRF7 can therefore be activated by ligation of the TLRs that utilise TRIF as an adaptor, namely TLR3 and TLR4. Interestingly, in murine macrophages, IRF3 can be activated by both LPS and the dsRNA analogue poly(I:C), which mimic bacterial and viral infection of TLR4 and TLR3, respectively; however in human macrophages, only LPS [not poly(I:C)] seems to induce IFNβ via IRF3 (Reimer et al. 2008; Coccia et al. 2004). This suggests that in human macrophages, poly(I:C) activates alternative IRF3-independent pathways to those activated in murine macrophages in order to induce Type I IFN gene expression.

The TLR3 receptor is activated by viral pathogen-associated molecular patterns (PAMPs) such as dsRNA, which is derived either directly from dsRNA viruses or from the replication intermediates of single-stranded RNA (ssRNA) viruses. TLR3 signalling is via the myeloid differentiation primary response gene 88 (MyD88)-independent pathway, which is dependent on the adaptor protein TRIF as well as TBK1, as Type I IFN production is impaired in macrophages from *Trif*$^{-/-}$ and *Tbk1*$^{-/-}$ mice stimulated with poly(I:C) (Perry et al. 2004; Yamamoto et al. 2003). TRAF3 forms a complex with both TRIF and TBK1, which positively regulates TRIF-dependent *Ifnb1* gene induction (Hacker et al. 2006). A complete immune response following TLR3 ligation requires both IRF3 and IRF7, as some gene expression is still observed in *Irf3*$^{-/-}$ cells stimulated with poly(I:C) (Honda and Taniguchi 2006).

The TLR4 cell-surface receptor is activated by PAMPs such as bacterial LPS or viral components including the RSV fusion (F) protein. TLR4 signalling leads to induction of the *Ifnb1* gene, but not the *Ifna* genes, suggesting that this pathway activates IRF3 rather than IRF7, as *Irf3*$^{-/-}$ cells do not produce IFNβ upon LPS stimulation whereas *Irf7*$^{-/-}$ cells exhibit a normal phenotype (Honda et al. 2005; Sakaguchi et al. 2003). Like TLR3, TLR4 signalling to *Ifnb1* predominantly occurs via the MyD88-independent pathway, utilising the adaptor proteins TRIF and TRAM (TRIF-related adaptor molecule) to activate TBK1, as *Ifnb1* gene induction is also completely abolished in *Tbk1*$^{-/-}$ macrophages stimulated with LPS (Perry et al. 2004). TLR4 activation by LPS also leads to the induction of pro-inflammatory cytokines via the MyD88-dependent pathway and the transcription factors IRF5/NFκB, as discussed later in the chapter.

The cytosolic pathway is initiated when viral PAMPs, such as dsRNA, are detected intracellularly by the cytosolic receptors RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma-differentiation-associated gene 5) via the C-terminal RNA-helicase domains of these receptors (Yoneyama et al. 2004, 2005). Once the dsRNA interacts with RIG-I/MDA5, the complex moves toward the mitochondria where RIG-I/MDA5 interact with their adaptor protein MAVS (mitochondrial antiviral signalling protein) via reciprocal CARD (caspase recruitment domain) domains, present at the C-terminus of RIG-I/MDA5 and N-terminus of MAVS (Kawai et al. 2005). This leads to an indirect activation of TBK1, via the protein kinase TRAF3 (Oganesyan et al. 2006), resulting in subsequent phosphorylation of IRF3 and IRF7 to induce Type I IFNs, as well as recruitment of the signalling proteins FADD, RIP1 and TRAF6 to the C-terminal effector domain of MAVS, which leads to activation of NFκB and proinflammatory cytokine gene induction (Seth et al. 2005). The integral role of MAVS in this process is demonstrated by impaired

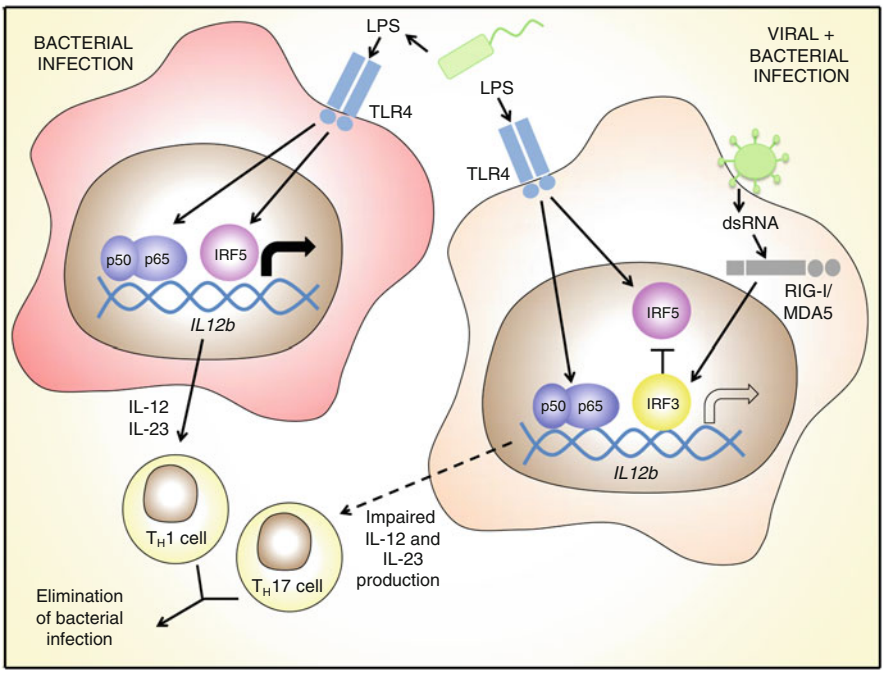

Fig. 22.2 Suppression of *IL12b* expression by IRF3 via RIG-I signalling. TLR signalling during a bacterial infection causes nuclear translocation of NFκB and IRF5, which activate the *IL12b* gene. Activation of RIG-I/MDA5 by dsRNA during a viral infection leads to the activation of IRF3, which inhibits IRF5 recruitment to the *IL12b* locus. This results in reduced production of the p40 subunit of IL-12 and IL-23, cytokines which are important for T_H1 and T_H17 differentiation and elimination of bacterial infection. Figure adapted from Negishi et al. (2012)

RIG-I/MDA5-mediated Type I IFN production in macrophages from MAVS-deficient mice (Sun et al. 2006).

Of interest, RIG-I activated IRF3 suppresses *IL12b* gene transcription via interfering with binding of another IRF family member, IRF5, at the gene promoter (Negishi et al. 2012) (see Fig. 22.2). This results in attenuated IRF5-driven T-helper type 1 (T_H1) and IL-17-producing T-helper (T_H17) cell differentiation (see below), and consequently in the death of mice at sub-lethal doses of bacterial infection (Negishi et al. 2012).

22.4 IRF8: A Lineage-Defining Factor for Macrophages

IRF8, also known as interferon consensus sequence binding protein (ICSBP), was originally identified as a nuclear protein that binds to major histocompatibility complex (MHC) class I genes (Driggers et al. 1990). IRF8 is expressed in cells of the

monocyte/macrophage lineage, as well as in B-cells and activated T-cells. *Irf8* mRNA expression is inducible by IFNγ, via STAT1 binding to an IFNγ activation site (GAS) in the *Irf8* promoter (Kanno et al. 1993). IRF8 is also synergistically induced by IFNγ in combination with LPS, leading to interactions with TRAF6 (Zhao et al. 2006), but not MyD88 (Negishi et al. 2005) downstream of TLR signalling, which suggests a role for IRF8 in the cytoplasm. Notch and RBP-J have also been recently described as regulators of IRF8 protein synthesis via the MNK1-eIF4E axis downstream of TLR4 signalling in macrophages (Xu et al. 2012) (see Fig. 22.3). This leads to enhanced expression of a subset of M1-phenotypic genes (see IRF4/IRF5 section below).

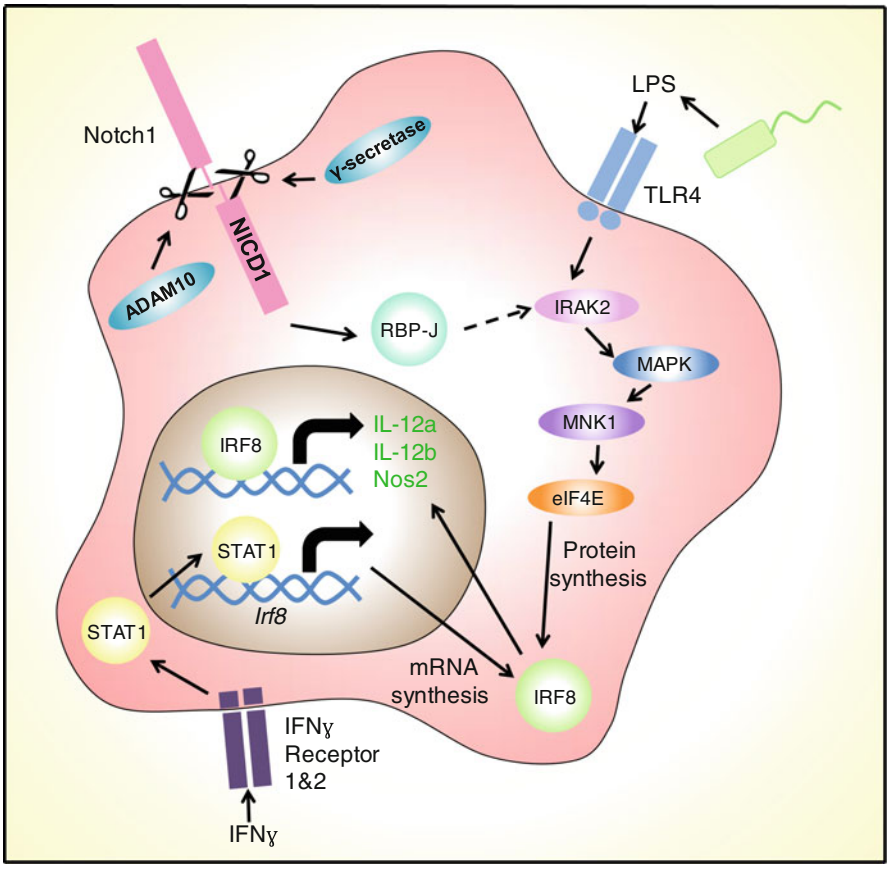

Fig. 22.3 Regulation of IRF8 protein synthesis and M1 macrophage polarisation by Notch signalling and RBP-J. Notch1-RBP-J signalling, which occurs via the enzymatic activities of ADAM10 and γ-secretase, is essential for TLR4-induced expression of a subset of M1 macrophage markers. RBP-J augments the activity of the MNK1-eIF4E axis, via IRAK2 and MAPK downstream of TLR4, to promote IRF8 protein synthesis. Subsequently, IRF8 drives the expression of a subset of M1 genes such as *IL12a*, *IL12b* and *Nos2*. RBP-J is also able to suppress M2 marker gene induction, including the gene *Jmjd3*, which in turn regulates IRF4 expression (a key factor in M2 macrophage polarisation)

22.4.1 IRF8 Drives Macrophage Differentiation

In macrophages, IRF8 is critical for regulation of myeloid cell growth and differentiation. *Irf8*$^{-/-}$ mice harbour increased numbers of common myeloid progenitor (CMP) cells, which are hyper-responsive to both GM-CSF (granulocyte-macrophage colony-stimulating factor) and G-CSF (granulocyte colony-stimulating factor), and display a strong reduction in their response to M-CSF (macrophage colony-stimulating factor) (Scheller et al. 1999). As a result, *Irf8*$^{-/-}$ myeloid progenitors give rise predominantly to granulocytes and few macrophages, even in the presence of M-CSF (Scheller et al. 1999). Accordingly, there is a reduction in the number of bone marrow cells expressing F4/80 and the M-CSF receptor in *Irf8*$^{-/-}$ mice (Scheller et al. 1999), potentially due to accumulation of c-Cbl in the absence of IRF8, which acts as a ubiquitin ligase to target activated M-CSF receptor for degradation (Kallies et al. 2002). Interestingly, the *Nf1* gene (Ras-GFP Neurofibromatosis 1), which encodes a protein that inactivates Ras in haematopoietic cells, is reported to be a direct target of IRF8; and *Nf1*$^{-/-}$ mice exhibit myeloproliferative symptoms due to a hypersensitivity to GM-CSF (Zhu et al. 2004; Huang et al. 2006). A lack of IRF8 transcript has been observed in cells from human patients with chronic myelogenous leukaemia (CML) (Schmidt et al. 1998), a disease that is characterised by a marked increase in granulocytes. The *Irf8*$^{-/-}$ mice also suffer from a syndrome that resembles human CML (Holtschke et al. 1996). Consistent with this, *Irf8* expression declines as CMPs differentiate into granulocytes, and persists in differentiating macrophages, where IRF8 in complex with PU.1 regulates the expression of numerous macrophage target genes, including *Prdm1* and *Ctsc* (Schmidt et al. 1998; Tamura et al. 2005). This demonstrates that IRF8 acts as a lineage-selecting factor, and drives CMPs to differentiate into mature macrophages, whilst inhibiting granulocytic growth and differentiation (Tamura et al. 2000).

22.4.2 Co-operative Regulation of Gene Expression by IRF8

IRF8 has been shown to both drive and inhibit expression of target genes. Despite possessing the conserved IRF DBD, IRF8 usually only binds to DNA when it interacts with other transcription factors via its C-terminal IAD, such as IRF1, IRF2 or PU.1. Conformational changes in IRF8 that occur when such interactions take place facilitate enhanced binding of both interaction partners to the DNA (Meraro et al. 1999). In complex with IRF1 or IRF2, IRF8 forms inhibitory complexes that repress IFNα- and IFNβ-inducible genes in macrophages such as *Isg15* by binding to an ISRE. In contrast, when IRF8 (together with IRF1 and/or IRF2) interacts with PU.1 to bind to an IRF-Ets composite sequence (IECS), the complex induces expression of myeloid target genes such as *Nox2* (Eklund et al. 1998), *Ncf2* (Eklund and Kakar 1999), *IL18* and *IL12b* (Kim et al. 1999). As a consequence, *Irf8*$^{-/-}$ macrophages are defective in the production of IL-12p40 (Giese et al. 1997), nitric oxide and reactive oxygen species (Fehr et al. 1997), thus have impaired development of T_H1 responses

and are highly susceptible to infection by multiple pathogens, including *Listeria monocytogenes* (Fehr et al. 1997), *Yersinia enterocolitica* (Hein et al. 2000) and *Toxoplasma gondii* (Scharton-Kersten et al. 1997). Interestingly, once activated by IFNγ and STAT1, IRF8 as part of a multi-protein complex can also bind to IFNγ activation sites (GAS) and regulate a second wave of transcription from GAS-promoters, therefore macrophages from *Irf8*$^{-/-}$ mice are also defective in the induction of some IFNγ-induced genes, even though STAT1 activation is still intact (Contursi et al. 2000). As a result of working with co-factors, IRF8 is therefore able to bind to a variety of DNA motifs. Interestingly, more evidence is gradually appearing that shows IRF8 is also able to bind directly to DNA, without the need of co-factors, for example genome-wide studies in macrophages and THP-1 cells have shown IRF8 binding to MHC Class I and II gene loci, and to the *Irf9* locus independently of PU.1 (Marquis et al. 2011; Kubosaki et al. 2010).

22.5 IRF4 and IRF5: Master Regulators of M2 and M1 Macrophage Polarisation

Macrophages are functionally polarised into M1 or M2 cells in response to infection by microorganisms, in order to drive the appropriate immune response. M1 macrophages are essential for clearing bacterial, viral and fungal infections, whereas M2 macrophages have an important role in responses to parasite infection, tissue remodelling, angiogenesis and tumour progression (Lawrence and Natoli 2011). Differentiation of monocytes into M1 macrophages can be induced by the growth factor GM-CSF or IFNγ, whereas M2 macrophage differentiation occurs in response to M-CSF, IL-4 or IL-13. Characteristic markers of the M1 phenotype include *Nos-2* (iNOS), *Tnf*, *IL12a*, *IL12b, IL6* and *IL23a* whilst M2 macrophages express *IL10*, *Arg1*, *Chi3l3*, *Fizz1*, and *Mrc1*. IRF5 and IRF4 have been identified as important factors in driving the expression of M1- and M2-related genes, respectively, and to this effect have mirroring transcriptional roles in macrophage polarisation. Interestingly, recent ChIP-seq studies have shown that IRF4 is recruited to the *Irf5* promoter in murine bone marrow-derived dendritic cells (Garber et al. 2012). Taking into account that IRF4 was shown to negatively regulate *Irf5* promoter reporter activities (Xu et al. 2011), this may represent a direct mechanism of regulating macrophage polarity by inhibition of *Irf5* gene expression.

22.5.1 IRF4 Regulates Both Macrophage Differentiation and M2 Polarisation

IRF4, also known Pip (PU.1 interacting partner) and ICSAT (interferon consensus sequence binding protein for activated T-cells), is expressed in immune cells including B-cells, T-cells and macrophages (Marecki et al. 1999). Like IRF3, activation of

macrophages by LPS or IFNγ does not induce *Irf4* expression, rather it induces conformational changes in the latent IRF4 protein to unmask its nuclear localisation sequence and drive nuclear translocation (Marecki et al. 1999). When bound to ISRE motifs, IRF4 functions as a transcriptional repressor, such as in co-operation with IRF8 at the *Isg15* promoter in macrophages (Rosenbauer et al. 1999). Like other members of the IRF family, however, IRF4 can also form activating transcriptional complexes, such as with PU.1. For example, IRF4/PU.1 binds to an IECS in an upstream enhancer region of the *IL1b* gene in macrophages, which is inducible by LPS (Marecki et al. 2001).

Ectopic expression of IRF4 or IRF8 (which exhibit approximately 70 % sequence homology) in myeloid progenitor cell lines leads to morphological changes consistent with differentiation into macrophages, including expression of CD11b, F4/80 and M-CSF receptor, and strong phagocytic activity (Yamamoto et al. 2011). Interestingly, *Irf4*$^{-/-}$ mice do not exhibit an increase in granulocyte number despite IRF4 (like IRF8) inhibiting the expression of the transcription factor *Cebpe*, which is essential for neutrophil differentiation (Yamamoto et al. 2011). Yet a lack of both IRF4 and IRF8 (*Irf4*$^{-/-}$*Irf8*$^{-/-}$ double knock-out mice) seems to severely augment granulocyte differentiation and diminish macrophage numbers to a greater extent than observed in *Irf8*$^{-/-}$ single knock-out mice. Concordant with this, *Irf4*$^{-/-}$*Irf8*$^{-/-}$ mice suffer a more severe form of CML than *Irf8*$^{-/-}$ mice (Yamamoto et al. 2011), and *Irf4* transcript levels are also reduced in human CML patients (Schmidt et al. 2000). This suggests that although IRF4 is important for macrophage differentiation from myeloid progenitors, its role is to co-operate with IRF8, which is the predominant factor driving this process.

The fact that *Irf4* expression is induced during macrophage differentiation (*Irf8* in comparison is highly expressed in myeloid progenitors) also suggests a more secondary role for IRF4 during these early stages. To this effect, an important role for IRF4 has been identified in the polarisation of macrophages toward an M2 phenotype, which occurs following differentiation of myeloid progenitors into macrophages and migration into tissues. Both chitin-induced (in vivo) and M-CSF differentiated bone marrow-derived (in vitro) macrophages from *Irf4*$^{-/-}$ mice have severely reduced expression of characteristic M2 macrophage markers, including *Arg1*, *Chi3l3*, *Fizz1* and *Mrc1* (Satoh et al. 2010). Consistent with this, ectopic expression of IRF4 in *Irf4*$^{-/-}$ bone-marrow macrophages can rescue expression of these characteristic M2 markers (Satoh et al. 2010). This therefore indicates that IRF4 specifically regulates macrophage polarisation to an M2 phenotype. As macrophage M1–M2 plasticity is a fine balance, the reduced expression of M2 markers observed in the absence of IRF4 also means an increase in M1 markers, including TNF, IL-12 and IL-6, resulting in high susceptibility of *Irf4*$^{-/-}$ mice to LPS-induced endotoxic shock due to increased levels of pro-inflammatory cytokines (Honma et al. 2005). Similarly, under more natural circumstances, the microRNA miR-125b can inhibit expression of *Irf4* in macrophages, leading to an activated phenotype consistent with M1 polarisation (Chaudhuri et al. 2011). It can therefore be said that IRF4 also negatively regulates the production of proinflammatory cytokines in response to TLR stimulation. This is profoundly different to IRF8, which positively regulates the proinflammatory cytokine IL-12, showing that despite the high

similarity of the IRF4 and IRF8 proteins, lower sequence homology at the C-terminus of IRF proteins can lead to diverse transcriptional effects. Consistent with a shift towards an M1 phenotype, *Irf4*$^{-/-}$ mice also exhibit faulty T_H2 responses during *Nippostrongylus brasiliensis* helminth infection (Zheng et al. 2009), for which defective M2 macrophages could be a contributing factor.

The *Irf4* gene itself is regulated by the histone demethylase JMJD3 (Jumonji domain containing-3), as H3K27 is differentially methylated at the *Irf4* locus in *Jmjd3*$^{-/-}$ compared to wild-type macrophages (Satoh et al. 2010). As H3K27 trimethylation is an epigenetic mark for silenced gene expression, demethylation of H3K27 at the *Irf4* locus by JMJD3 drives *Irf4* expression. This demethylase activity is dependent upon the DNA-binding ability of JMJD3, indicating that *Irf4* is a JMJD3 target gene. Chitin-elicited *Jmjd3*$^{-/-}$ macrophages are themselves deficient in *Arg1*, *Chi3l3*, *Fizz1* and *Mrc1*, as are *Jmjd3*$^{-/-}$ macrophages isolated from the lung following infection with *N. brasiliensis* (Satoh et al. 2010). Interestingly, ectopic expression of IRF4 in *Jmjd3*$^{-/-}$ M-CSF differentiated bone-marrow macrophages also upregulates expression of M2 markers, which confirms that IRF4 is active in M2 macrophage polarisation downstream of JMJD3 (Satoh et al. 2010).

22.5.2 IRF5 Regulates M1 Macrophage Polarisation

IRF5 is expressed in immune cells including B-cells, dendritic cells and macrophages, and is activated by multiple TLRs and their corresponding ligands (Takaoka et al. 2005). In particular, a significant fraction of IRF5 becomes detectable in the nucleus post-LPS, -ssRNA and -CpG DNA stimulation of murine macrophages, supporting the fact that TLR4, TLR7 and TLR9 invoke IRF5 nuclear translocation (Takaoka et al. 2005). Like many of the IRF family members, post-translational modifications are crucial to switch IRF5 from autoinhibition to its active conformation. In particular, phosphorylation of IRF5 at Ser425/427/430 is required for NDV-induced activation (Barnes et al. 2002), which involves unfolding of the C-terminus of IRF5 to make the IAD accessible for dimerisation and protein–protein interactions. Phosphorylation of Ser430 in particular is important for the structural changes that allow IRF5 to interact with the co-activator CBP (Chen et al. 2008). IRF5 can be phosphorylated by IKKε and TBK1, like IRF3 and IRF7, however TBK1 and IKKε do not seem to induce nuclear translocation of IRF5, so currently the kinases that activate IRF5 in vivo remain elusive (Lin et al. 2005).

Irf5$^{-/-}$ macrophages exhibit severely impaired induction of proinflammatory cytokines, a major contributing factor to the observed resistance of *Irf5*$^{-/-}$ mice to LPS- and CpG-induced endotoxic shock (Takaoka et al. 2005), in stark comparison with the susceptibility observed by *Irf4*$^{-/-}$ mice. The *Irf5*$^{-/-}$ phenotype includes a marked decrease in both mRNA and serum protein levels of IL-6, TNF and IL-12p40 in response to TLR activation, which therefore suggests direct transcriptional activation of these genes by IRF5 (Takaoka et al. 2005). This is confirmed by the presence of ISREs in many proinflammatory gene promoters, including *Tnf*, *IL12a*, *IL12b*, *IL23a* and *IL6* to which IRF5 can bind (Takaoka et al. 2005;

Krausgruber et al. 2011). Interestingly, IRF5 is also recruited to the promoter of the M2 marker, *IL10*, in M1 macrophages, where it seems to prevent recruitment of RNA polymerase II and inhibit *IL10* gene induction (Krausgruber et al. 2011). IRF5 therefore exhibits diverse modes of transcriptional activity, like many other members of the IRF family, so the recruitment of IRF5 co-factors is likely to be an important factor in the ability of IRF5 to both positively and negatively regulate gene expression. In this way, IRF5 is able to directly polarise macrophages toward a proinflammatory M1 phenotype—the opposite activity of IRF4—and as a result can polarise T-cells toward T_H1 and T_H17 responses (Krausgruber et al. 2011) (see Fig. 22.4). Consistent with this, genetic polymorphisms in the human *Irf5* gene

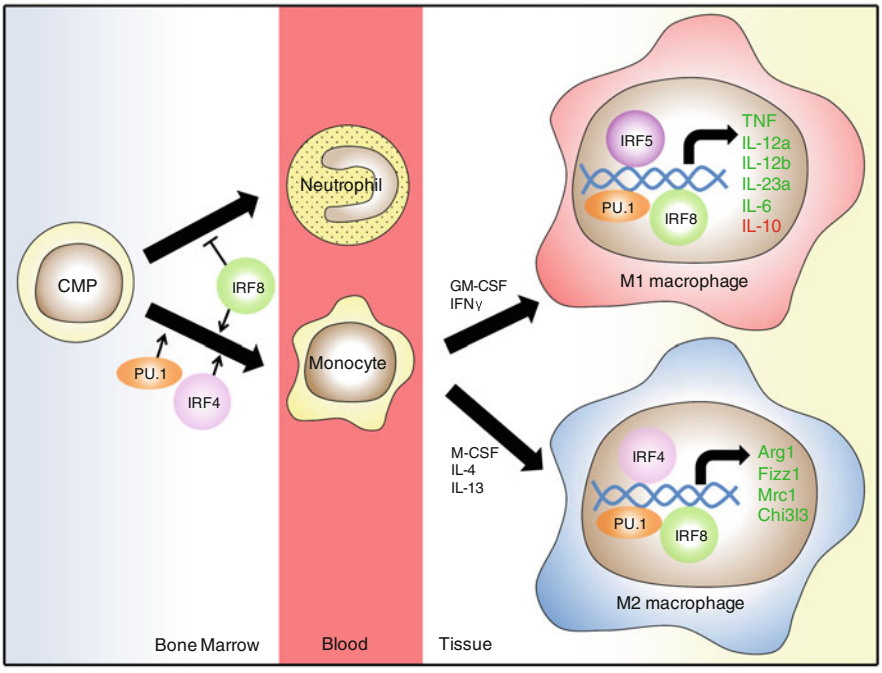

Fig. 22.4 The role of IRFs in macrophage polarisation. Common myeloid progenitor (CMP) cells in the bone marrow are differentiated to peripheral blood mononuclear cells, including monocytes and neutrophils, which migrate from the bone marrow to circulate in the blood. Differentiation of CMPs into monocytes is driven by the lineage-defining factors PU.1 and IRF8, in combination with IRF4. In contrast, IRF8 inhibits the differentiation of CMPs into neutrophils, thus polarising differentiation of CMPs toward monocytes/macrophages. Monocytes in the blood migrate into tissues where they come into contact with growth factors, which are produced in the tissue environment by local cells. Under normal homeostatic conditions, monocytes come into contact with M2-driving factors such as M-CSF, IL-4 and IL-13, leading to their differentiation to M2 macrophages. The expression of phenotypic M2 markers is driven by IRF4, and inhibited by IRF5. Under inflammatory conditions, monocytes come into contact with M1-driving factors such as GM-CSF and IFNγ, which are produced locally by immune cells responding to the infection, thus leading to M1 macrophage differentiation. The expression of phenotypic M1 markers is driven by IRF5. PU.1 and IRF8 act as markers for IRF4/IRF5 recruitment at the promoters/enhancers of characteristic macrophage marker genes

that lead to higher expression or the expression of alternative isoforms of IRF5 have been positively associated with autoimmune diseases such as SLE, RA, MS and IBD (Dideberg et al. 2007; Dawidowicz et al. 2011; Kristjansdottir et al. 2008; Graham et al. 2006), where T_H1 and T_H17 cells contribute to disease pathogenesis. Conversely, the *Irf5* gene has been negatively associated with asthma, a T_H2-driven disease (Wang et al. 2012).

Interestingly, many of the pro-inflammatory genes regulated by IRF5 also contain binding sites for NFκB in proximity to their promoters, which infers that IRF5 cooperatively regulates these genes in combination with NFκB (Takaoka et al. 2005). This makes sense, as NFκB is the universal regulator of inflammation downstream of all the TLRs. Further investigation at the *Tnf* locus in particular has revealed two modes of transcriptional action of IRF5: both direct binding to ISREs in the *Tnf* 5′ region, and indirect binding of IRF5 to the 3′ of *Tnf* via protein–protein interactions with the NFκB subunit RelA at a κB site (Krausgruber et al. 2010). Knockdown of IRF5 in M1 macrophages reduces *Tnf* gene induction upon LPS-stimulation down to the levels observed in M2 macrophages, which only express very low levels of IRF5 (Krausgruber et al. 2010). This suggests that a role of IRF5 in M1 macrophages is to synergistically boost expression of a subset of NFκB regulated genes, to levels above those observed in M2 macrophages; which poses an opportunity for therapeutic intervention to reduce such exaggerated cytokine production in autoimmune disease by targeting IRF5 rather than the broader activity of NFκB.

22.5.3 IRF4 and IRF5 Compete to Interact with the Adaptor MyD88 During TLR Signalling

IRF5 is differentially expressed in polarised macrophages, with high levels of expression in M1 macrophages and low expression in M2 macrophages, whereas IRF4 expression remains the same throughout polarisation. This suggests that in order to mediate its proinflammatory transcriptional activity, IRF5 needs to overcome the presence of IRF4 and the "default" setting of an M2 macrophage phenotype.

IRF5 can be activated by the TLR MyD88-dependent signalling pathway, for example in response to TLR9 activation, when IRF5 interacts with both MyD88 and TRAF6, which facilitates activation and translocation of IRF5 to the nucleus to induce ISRE-containing gene promoters (Takaoka et al. 2005). Interestingly, IRF4 is also activated by the MyD88-dependent pathway, and can directly interact with MyD88 (Negishi et al. 2005). The fact that *Irf4*$^{-/-}$ mice show enhanced activation of NFκB and JNK in response to LPS, as well as enhanced proinflammatory cytokine production, highlights the fact that IRF4 negatively regulates innate immune responses by negatively modulating TLR signalling pathways. Both IRF5 and IRF4 have been shown to bind to the same region of the MyD88 protein, which suggests

that this is the point of competition for polarising activity, therefore TLR-induced IRF5 competes for MyD88-binding during inflammation, which leads to M1 macrophage polarisation and a proinflammatory response.

22.6 Future Perspectives

It is clear that many members of the IRF family are integral to macrophage differentiation and function, both in the regulation of anti-viral Type I IFN responses and in the polarisation of macrophages toward a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. The ultimate test and validation of their role must, however, come from the studies of infection and inflammatory disease models in animals with macrophage-specific ablation of IRF proteins, which are currently in deficit (see Table 22.1).

It is also evident that the IRF family members prefer to co-operate with other factors in order to mediate their functions, including with other members of the IRF family via the formation of active heterodimers, and also with non-related co-factors and co-activator proteins. The strongest evidence for such co-operation comes from the NFκB family of transcription factors, which like the IRFs are activated downstream of the TLRs. Both the IRF and NFκB transcription factors are integral for the induction of many of the same immune gene targets in macrophages, and co-operate with each other by protein–protein interactions, such as at the *Ifnb1* and *Tnf* gene loci, in order to boost target gene expression. There is also increasing evidence that members of the IRF family enjoy co-operating with members of the TRIM family in order to regulate IRF activity, both at the level of chromatin modification (TRIM28 with IRF5) and post-translational regulation (TRIM59/TRIM21 with IRF3/IRF7) (Kondo et al. 2012; Higgs et al. 2008; Kong et al. 2007; Eames et al. 2012). Future research on IRF family members should therefore be focussed, on the one hand, on proteomic studies, which will be informative for determining IRF function based on the protein–protein interactions they perform.

On the other hand, and also in parallel with proteomic studies, high-throughput genomic studies that identify IRF target genes will also be extremely beneficial in ascertaining the full repertoire of genes that an IRF protein regulates. For example, several IRF genome-wide ChIP-seq studies in myeloid cells have already been performed (see Table 22.1), which will give us a wealth of information about IRF function in terms of the groups of genes that they regulate. Also, by overlaying different ChIP-seq data sets, we will have a better understanding of the IRF transcriptional network and its co-operation with other transcription factor families in response to a given stimuli. This may lead to potential for therapeutic intervention, such as preventing co-factor binding using small molecule inhibitors. The fact that IRF5, for example, only targets a specific subset of genes downstream of TLR activation already highlights the exciting possibility of specifically targeting IRF pro-inflammatory function, in comparison with inhibition of NFκB activity which would be more detrimental to general macrophage function due to the more broad activities of NFκB.

Table 22.1 Availability of IRF knock-out mice and genome-wide studies

IRF	KO design	Availability	Location (source)	Macrophage-related KO phenotype	Macrophage microarrays	Myeloid genome-wide data sets
IRF1	(1) DBD targeted systemic KO	Mouse	JAX (Tak Mak)	Impaired induction of IL-12, iNOS and IFNβ by macrophages upon viral stimulation (Taki et al. 1997; Lohoff et al. 1997; Negishi et al. 2006)	IRF1 KO (peritoneal macrophages) (Dror et al. 2007)	ChIP-seq BMDCs (Garber et al. 2012)
	(2) Floxed (LoxP sites flanking Exon 3)	ES cells	WTSI			ChIP-seq PBMCs (Shi et al. 2011)
IRF2	(1) DBD targeted systemic KO	Mouse	Tak Mak lab	Impaired induction of IL-12 by macrophages (Salkowski et al. 1999)	N/A	ChIP-seq BMDCs (Garber et al. 2012)
IRF3	(1) DBD targeted systemic KO	Embryos	RBRC	Defective Type I Interferon production in by macrophages upon LPS/viral stimulation (Sato et al. 2000)	IRF3 KO (dendritic cells and peritoneal macrophages) (Ogawa et al. 2005; Lazear et al. 2013)	N/A
		Mouse	Taniguchi lab			
IRF4	(1) Systemic KO	Mouse	Tak Mak lab	Regulates expression of M2 macrophage markers (Satoh et al. 2010)	IRF4 KO (bone marrow-derived macrophages (El Chartouni et al. 2010)	ChIP-seq BMDCs (Garber et al. 2012)
	(2) Floxed/GFP (LoxP sites flanking Exon 1 and 2)	Mouse	JAX (Ricardo Dalla-Ferrara)	High susceptibility to LPS/CpG-induced endotoxic shock (Honma et al. 2005)		
IRF5	(1) Exon 2 targeted systemic KO	Mouse	Taniguchi/Mak labs	Regulates expression of M1 macrophage markers (Krausgruber et al. 2010, 2011)	IRF5 overexpression (bone marrow-derived macrophages) (Krausgruber et al. 2011)	ChIP-seq PBMCs (Wang et al. 2013)
	(2) Floxed (LoxP sites flanking Exon 2)	Mouse	JAX (Paula Pitha)	Resistant to LPS/CpG-induced endotoxic shock due to reduced cytokine levels in serum (Takaoka et al. 2005)	IRF5 KO (dendritic cells) (Lazear et al. 2013)	

IRF6	(1) Floxed (LoxP sites flanking Exon 3)	ES cells	WTSI	N/A	N/A	N/A
IRF7	(1) Exon 1/2 targeted systemic KO	Mouse	RBRC (Tadatsugu Taniguchi)	Defective Type I Interferon production in by macrophages upon viral stimulation (Sato et al. 2000)	IRF7 KO (dendritic cells) (Lazear et al. 2013)	N/A
	(2) Floxed (LoxP sites flanking Exon 2)	ES cells	KOMP (Welcome Trust Sanger Institute)			
IRF8	(1) Exon 2 targeted systemic KO	Embryo	EM (Ivan Horak)	Impaired macrophage differentiation, suffer from CMP (Scheller et al. 1999)	IRF8 KO (peritoneal macrophages) (Dror et al. 2007)	ChIP-Chip THP-1 (Kubosaki et al. 2010)
	(2) Floxed (LoxP sites flanking Exon 2)	Mouse	JAX (Herbert Morse III)	Defective IL-12 and iNOS production by macrophages (Giese et al. 1997; Fehr et al. 1997)	IRF8 KO (myeloid progenitor cells) (Tamura et al. 2005)	ChIP-Chip human macrophages (Marquis et al. 2011)
IRF9	(1) Exon 1/2 targeted systemic KO	Embryos	RBRC (Tadatsugu Taniguchi)	N/A	N/A	N/A

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Chapter 23
Kruppel-Like Factors in Monocyte–Macrophage Biology

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23.1 Introduction

Kruppel-like factors (KLFs) are a subfamily of the zinc-finger class of DNA-binding transcriptional regulators that bear homology to the *Drosophilia melanogaster* Kruppel protein. The name Kruppel is derived from the German word meaning "cripple" and is given to an early developmental fly gene based on the observation that Drosophilia embryos deficient in the Kruppel gene developed abnormal thoracic and abdominal segmentation and appeared "crippled" (Nusslein-Volhard and Wieschaus 1980; Jackle et al. 1985; Zuo et al. 1991).

Certain key features distinguish KLFs from other members of the family of zinc-finger transcription factors. Primarily, KLFs possess three $Cysteine_2/Histidine_2$ containing zinc fingers at the C-terminus of the protein (Turner and Crossley 1999; Bieker 1996). The interfinger space contains a highly conserved seven residue sequence [TGEKP(Y/F)X]. Finally, KLFs bind consensus DNA sequences such as CACCC elements or GT box on target genes that is enabled by three critical residues within each zinc finger (Bieker 1996; Dang et al. 2002). Individual members of the KLF family differ from each other in the non-DNA-binding regions that are highly divergent and affect transactivation and transrepression.

Sp1, one of the first identified mammalian transcription factors (Kadonaga et al. 1987), also binds GC-rich DNA segments via three C_2H_2 zinc fingers. Since KLFs

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bear homology with this zinc-finger transcription factor, they are classified as the Sp1/KLF family and studies now identify the members of this family as regulators of a broad set of genes that affect development and biology of many tissue types (McConnell and Yang 2010). Importantly, the Sp/KLF family members affect each other's function or by competing for the same DNA-binding site (Dang et al. 2002; Black et al. 2001; Haldar et al. 2007; Zhang et al. 2010).

The first mammalian KLF (erythroid KLF or EKLF or KLF1) was discovered in 1993 as an essential regulator of globin gene synthesis in erythrocytes. Since then, a large body of data has been gathered elucidating the biological functions of the KLF factors in a broad spectrum of hematopoietic and non-hematopoietic cell types in health and disease. To date, a total of 17 mammalian KLFs have been identified (designated KLF1 through KLF17, numbered in the chronological order of identification). These transcription factors regulate various aspects of cellular development, proliferation, differentiation, and activation. Within the hematopoietic system, and in addition to the aforementioned role in erythrocytes, KLFs have been shown to serve as essential determinants of lymphoid and myeloid biology. With respect to the latter, studies over the past decade have implicated four members of this gene family—KLF1, KLF2, KLF3, and KLF4—in the context of monocyte/macrophage biology. Here, we review these findings and their implications with regard to myeloid biology.

23.2 Kruppel-Like Factor 1 (KLF1)

Montaner and colleagues (2004) first described KLF1 in macrophages, including its role in IL-12 p40 expression. Although KLF1 alters IL-12 p40 transcription in RAW264.7 cells by modulating NFκB binding activity in activated cells, no changes in NFκB activity were noted in resting cells. Since IL-12 p40 plays an important role in activation of innate immunity, bifunctional control of IL-12 p40 expression is potentially important. However, gain- and loss-of-function studies in vitro or in vivo are lacking and thus the physiological significance of macrophage KLF1 remains unknown.

23.3 Kruppel-Like Factor 2 (KLF2)

The role of KLF2 in hematopoietic biology was first described in thymocytes where it was shown to promote cellular quiescence and in its absence result in partial T-cell activation (Buckley and Evershed 2001). In mature thymocytes, naïve T cells and memory T cells, KLF2 was found to bind and transactivate the promoter of sphingosine-1-phosphate receptor-1 to promote thymocyte emigration from peripheral lymphoid tissues and recirculation through the lymphatic system (Carlson et al. 2006). Initial interest regarding the role of this factor in myeloid biology developed

from the observation that KLF2 levels were reduced in peripheral bone marrow cells of patients afflicted with both acute (sepsis) (Mahabeleshwar et al. 2012) and chronic (atherosclerosis) inflammatory diseases (Carlson et al. 2006). These findings coupled with the observation that KLF2 expression in myeloid cells was strongly attenuated with lipopolysaccharide (LPS) stimulation suggested that this factor may confer and anti-inflammatory or quiescent state, similar to its role in T-cells (Das et al. 2006).

Das et al. were first to demonstrate in vitro the anti-inflammatory nature of KLF2 in macrophages. Forced expression of KLF2 in macrophages abrogated LPS-mediated induction of proinflammatory factors and cytokines including COX-2, TNFa, IL-1b, and MCP-1 (Das et al. 2006; Atkins et al. 2008). Mechanistically, KLF2 was shown to inhibit the transcriptional activity of NFκB and AP-1 (Fig. 23.1) by impaired recruitment of the transcriptional coactivator complex p300/CBP-associated factor (PCAF) (Atkins et al. 2008; Makowski et al. 2001). Perhaps more

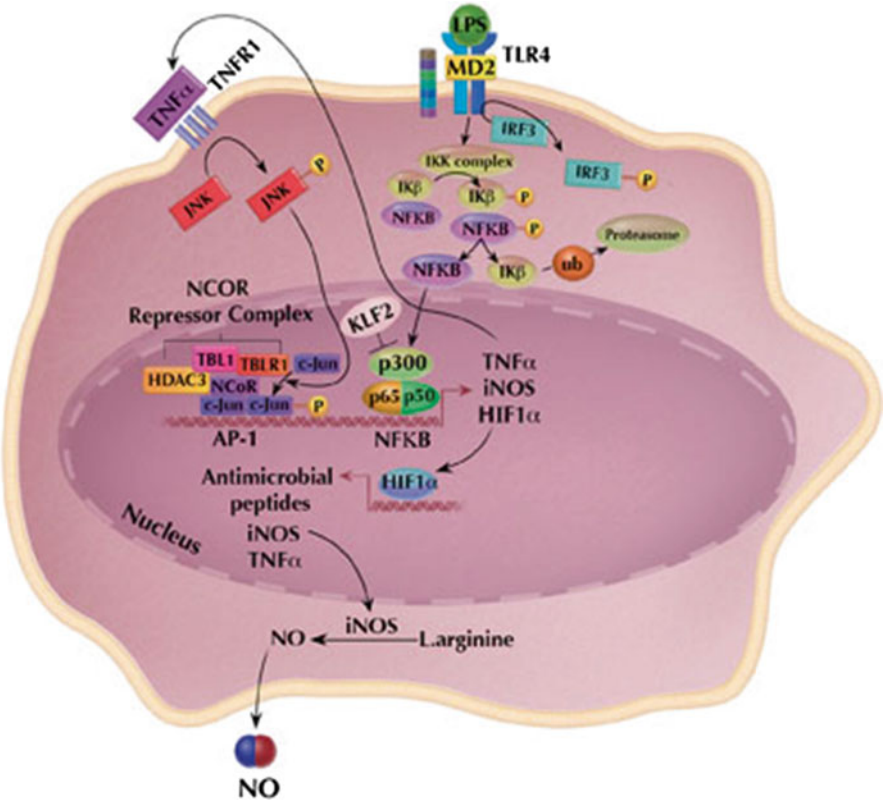

Fig. 23.1 Mechanistic basis for KLF2 in altering monocyte activity. *TNFR* TNF receptor, *LPS* lipopolysaccharide, *IRF* interferon-recognition factor, *IKK* Iκ kinase, *NFκB* nuclear factor kappa B, *HIF* hypoxia inducible factor, *NCoR* nuclear receptor co-repressor, *NO* nitric oxide

importantly, an anti-parallel effect was observed in loss-of-function studies (Mahabeleshwar et al. 2011). Mice bearing myeloid-specific deletion of KLF2 exhibited enhanced plasma levels of many inflammatory targets, an effect that was exaggerated in the setting of polymicrobial infection or endotoxin challenge (Mahabeleshwar et al. 2012). Strikingly, while mice showed enhanced survival and bactericidal activity in the face of polymicrobial sepsis, they experienced 100 % mortality with LPS exposure. These findings suggest that while enhanced macrophage activation was protective during bacterial infection, it was maladaptive and detrimental in the setting of overwhelming sepsis (Makowski et al. 2001; Mahabeleshwar et al. 2011). Mechanistically, the differential effect of KLF2 deficiency in these two models was linked to its ability to inhibit HIF-1 transcription by NFκB. Thus, KLF2 serves as a negative regulator of the ancient and conserved NFκB-HIF axis.

Animal models of chronic inflammation reveal that myeloid KLF2 regulates atherogenesis and arthritis. Atkins et al. showed that hemizygous deficiency of KLF2 results in severe atherosclerosis in ApoE$^{-/-}$ mice. In vitro, these macrophages exhibited enhanced LDL uptake in contrast to their KLF2-overexpressing counterparts (Atkins et al. 2008). Mechanistically, KLF2 targets the lipid binding protein aP2, whose deficiency in macrophages was previously shown to result in murine resistance to atherosclerosis (Makowski et al. 2001; Boord et al. 2002). In this model, attenuated KLF2 expression resulting in augmented aP2 expression likely leads to foam cell formation. In keeping with this finding, Lingrel and colleagues showed that myeloid-specific knockout of KLF2 augments atherogenesis in hypercholesterolemic Ldldr$^{-/-}$ mice. The mechanism underlying this finding is believed secondary to oxidative stress associated with augmented myeloid cell adhesion to endothelial cells, a consequence of enhanced CD11b expression on macrophages (Lingrel et al. 2012). Collectively, these observations highlight a central role for KLF2 as an essential repressor of macrophage inflammation.

More recently, an animal model of rheumatoid arthritis was demonstrated to be more severe in KLF2 hemizygous mice. Arthritis severity correlated with increased peripheral circulation and recruitment of inflammatory macrophages to the damaged joint. Additionally, bone marrow expression of key proteins that are involved in the development of arthritis was affected. Expression of heat shock proteins (HSP60 and HSP90) involved in T-cell activation, metalloproteinases (MMP13) and Akt associated with anti-apoptosis were enhanced whereas attenuated expression of pPTEN, p21, p38, and HSP25/27 was demonstrated. While the mechanism underlying these findings has not been elucidated, it is certainly possible that Akt-mediated upregulation of p65 results in binding of NFκB to the HSP90 promoter (Das et al. 2012). Interestingly, disease severity was associated with the differentiation of inflammatory monocytes to the osteoclast lineage, which contributes to degree of cartilage and bone damage in these mice (Das et al. 2012). These findings demonstrate that in both the acute and chronic inflammatory disease setting, KLF2 is requisite for maintenance of an anti-inflammatory phenotype.

23.4 Kruppel-Like Factor 3 (KLF3)

KLF3, like KLF1, was first cloned from erythroid tissue (Crossley et al. 1996). Considerable progress has since been made in understanding the molecular mechanisms by which this transcription factor alters gene expression. KLF3 mainly acts as a repressor of transcription and recruits the transcriptional co-repressor C-terminal binding protein (CtBP) to promoters of target genes (Turner and Crossley 1998). Studies further demonstrate that repression is also dependent on sumoylation (Perdomo et al. 2005).

Recent studies define a role for KLF3 in differentiation and knockout mice exhibit a myeloproliferative disorder with a phenotype similar to that of motheaten mice that bear mutations in the SHP-1 gene. Although the mechanism for the KLF3-mediated effect on myeloid cells remains to be elucidated, KLF3-deficient mice display reduced expression of SHP-1 phosphatase gene suggesting that it may be involved in pathways that activate this target (Tsui and Tsui 1994; Perkins et al. 1997).

23.5 Kruppel-Like Factor 4 (KLF4)

KLF4 is an important regulator of monocyte/macrophage differentiation and subset speciation. Early studies identified KLF4 expression in hematopoietic stem cells where its expression was augmented with monocytic differentiation. Consistent with this result, KLF4 overexpression resulted in Ly6C^{hi} and Ly6C^{lo} monocyte subspeciation (Feinberg et al. 2007; Alder et al. 2008).

KLF4, similar to KLF2, promotes an anti-inflammatory macrophage phenotype by inhibiting cytokine-mediated activation of macrophages through impaired NFκB activity. KLF4 sequesters essential NFkB coactivators, p300 and PCAF, thereby abrogating M1 polarization (Liao et al. 2011). However, KLF4 and KLF2 exhibit anti-parallel effects in the context of M2 differentiation. Liao et al. demonstrated that KLF4 is requisite for M2 polarization. IL-4 stimulated macrophages under basal conditions differentiate along the alternative pathway into M2 macrophages. These macrophages interestingly exhibit enhanced KLF4 expression. In vitro, IL-4 or IL-13 stimulation of KLF4-deficient macrophages resulted in impaired expression of typical M2 markers (Liao et al. 2011). Additionally, the absence of KLF4 resulted in increased expression of inflammatory genes and enhanced bactericidal activity. Mechanistic studies revealed that IL-4 induces STAT6 phosphorylation to promote KLF4 gene expression (Fig. 23.2). KLF4 in-turn cooperates with STAT6 to promote an M2 gene profile. Similar to STAT1 and STAT6, KLF4 serves as a tipping point in M1 versus M2 polarization; lack of KLF4 thus facilitates M1 polarization and impairs M2 polarization (Liao et al. 2011).

Murine studies have revealed that myeloid-specific KLF4 deficiency promotes an altered metabolic phenotype typical of metabolic syndrome that is characterized by glucose intolerance and insulin resistance. Furthermore, high-fat feeding renders

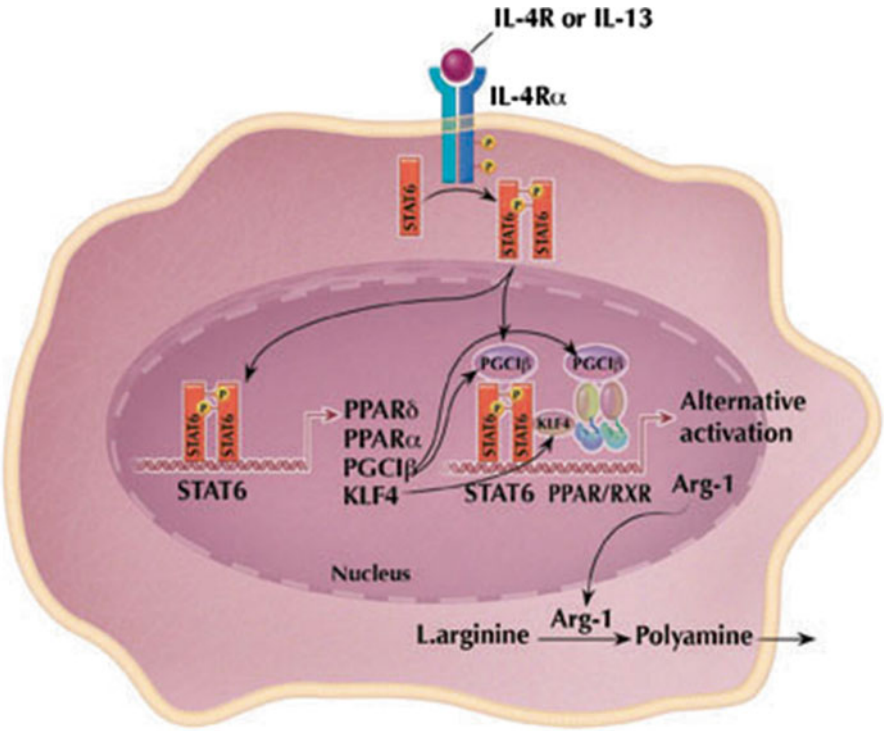

Fig. 23.2 Mechanisms involved by which KLF4 alters monocyte activity. *PGC1β* peroxisome proliferator-activated receptor gamma coactivator 1-β, *PPAR* peroxisome proliferator-activated receptor, *Arg-1* arginase-1, *STAT* signal transducers and activators of transcription, *IL-4Rα* interleukin-4 receptor alpha

these mice susceptible to obesity, diabetes, and atherosclerosis (Liao et al. 2011; Sharma et al. 2012). The metabolic phenotype of KLF4-null mice is a signature of M1 macrophage activation and impaired M2 macrophage activity. The clinical relevance of these findings was observed in human obese and diabetic patients. Obese patients typically develop a highly cellular adipose tissue compartment characterized by the infiltration of classically activated adipose tissue macrophages (ATMs) believed important mediators of insulin resistance and type 2 diabetes mellitus progression (Kang et al. 2008; Heilbronn and Campbell 2008). Obese subjects showed significantly lower KLF4 gene expression in visceral as well as subcutaneous adipose tissues than age-matched lean control subjects, an observation that was irrespective of diabetic status (Kang et al. 2008; Heilbronn and Campbell 2008). These observations strongly suggest that KLF4 is an important transcription factor requisite for M2 polarization and protection from a maladaptive metabolic and inflammatory phenotype.

23.6 Closing Remarks

Since the initial identification and characterization of the first KLF over 15 years ago new roles are constantly being discovered for members of this family in both health and disease. The studies discussed above highlight the significance of KLFs in the development and function of monocytes and macrophages, findings that bear important implications for a broad spectrum of disease states characterized by dysregulated inflammation. However, while the studies to date clearly link the KLFs gene family to the biology of myeloid cells, a number of additional questions merit investigation. For example, given the phylogenetic proximity of KLF2 and KLF4, the basis of the differences noted in the function of these two factors requires further study. While it is understood that members of the same family differ in their transactivation and repression domains, other issues such as relative expression levels of each factor, protein stability, interactions with other proteins, post-translational modifications such as acetylation, phosphorylation and sumoylation may determine macrophage/monocyte subspeciation and function in response to a particular stimulus or disease milieu. Further, several additional KLFs are expressed in myeloid cells but their function remains completely unknown (Liao et al. 2011). Assuming that multiple KLFs are expressed and function within the same cell, the relative importance of each of these KLFs under various physiological and disease states will need to be determined. Finally, there is strong evidence that members of the nuclear receptor (NR) family of proteins also regulate macrophage development and polarization (Lawrence and Natoli 2011; McMorrow and Murphy 2011; Olefsky and Glass 2010). Whether the KLFs and NR protein cooperate in some manner to orchestrate myeloid biology is an intriguing question worthy of pursuit.

Monocytes/macrophages play an integral role in innate and adaptive immunity with implications for both acute and chronic inflammatory diseases. Therefore, a better understanding of the factors that alter the function of these cells will provide the stepping stones for the development of targeted therapy that can potentially be utilized to manipulate these factors to maintain health and treat disease.

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Chapter 24
Regulation of Macrophage Polarization by the STAT–SOCS Signaling Axis

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24.1 Introduction

What is macrophage polarization? How does STAT–Suppressors Of Cytokine Signaling (SOCS) signaling regulate polarization? The goal of this review is to summarize current knowledge pertinent to the second question. However, before we review the complex cytokine-driven processes that both promote and then feedback to inhibit polarization, we need to address the first question. There are at least four broad definitions of macrophage polarization that stem from past and ongoing research. So far, there is no agreement on the nomenclature and definitions of polarization. Furthermore, terminology concerning polarization is loose, overlapping, redundant, and often contradictory. Here we will summarize the broad definitions of polarization and define the terms we will use through this chapter.

The most common macrophage polarization terms are M1 and M2, which are often used interchangeably with the descriptors "classically activated" macrophages (CAMs) and "alternatively activated" macrophages (AAMs). These terms generally represent two extremes and were based in part on the T_H1 and T_H2 polarization phenotypes of CD4+ T helper (T_H) cells. Ironically, we now know that CD4+ T cells are plastic, do not adopt fixed cytokine-producing characteristics, and encompass multiple other "subtypes." Mills et al. (2000) based the M1 and M2 polarization states of macrophages on the intrinsic arginine utilization profiles of macrophages from different mouse strains (as noted earlier by Modolell and colleagues) (Modolell

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et al. 1995; Munder et al. 1998, 1999). For example, B10D2 macrophages seemed to make more nitric oxide (NO) while Balb/c and DBA/2 macrophages made less NO and produced more ornithine as a result of increases in arginase activity (relative to C57BL/6 macrophages). A key claim of Mills was that the polarization states were intrinsic to macrophages because they were also observed on SCID backgrounds (lacking most lymphocytes) (Mills et al. 2000). This highly simplistic terminology was pervasive and soon expanded beyond the original definition. The extrapolation of macrophage activity in a lymphocyte-deficient background is difficult, if not impossible, to extend to normal immune responses which of necessity involve lymphocytes. In addition, C57BL/6 mice have a polymorphism in *Slc7a2*, which encodes the main arginine transporter expressed in macrophages, thus lowering their ability to shunt arginine to arginase I (Arg1) (Sans-Fons et al. 2013), a fact that was unknown at the time the Mills paper was published. Furthermore, the complex regulation of Arg1 in M1 macrophages (often incorrectly denoted as an M2 marker) was not appreciated (El Kasmi et al. 2008; Murray and Wynn 2011a; Qualls et al. 2010).

Gordon defined polarization in a far more rigorous way (Gordon 2003; Gordon and Martinez 2010). He proposed that "resting" macrophages stimulated with IL-4 or IL-13 adopted a state of "alternative activation" as opposed to macrophages stimulated with IFNγ or microbial products such as LPS (classical activation profiles, sometimes used interchangeably). Gordon further proposed another activation type termed "innate," where macrophages are exposed to microbial products prior to subsequent stimulation with T cell-derived polarizing cytokines. Gordon's definition has the advantage of defining what cytokines and microbial products a macrophage has been exposed to, and provides guidance on predictable gene expression profiles.

Mantovani, Mosser, and others then further subdivided the M1, M2 concept, to reflect gene expression profiles after stimulation (Biswas and Mantovani 2010; Mosser and Edwards 2008; Murray and Wynn 2011b). The problem with these definitions is translation to in vivo settings. Knowing that macrophages are plastic (Rutschman et al. 2001; Stout and Suttles 2004; Whyte et al. 2011) and can respond to concurrent stimuli (i.e., there is no "locked" gene expression profile that corresponds to the distinction between CD4+ and CD8+ T cells as an extreme example), the sub-division approach remains problematic. Ivashkiv developed the concept of polarization diversity further by building in examples of in vivo infection and inflammatory states (Ivashkiv 2012).

Finally, we note that some investigators use M1 and M2 polarization to refer to macrophages grown in CSF-1 or GM-CSF, respectively. In this system, authors attempt to tie CSF-1-derived macrophages to resident tissue macrophages (that are CSF-1 receptor-dependent) while inflammatory bone marrow-derived monocytic cells that mature to macrophages are partly GM-CSF-dependent. Hamilton and colleagues (Fleetwood et al. 2009; Lacey et al. 2012; Way et al. 2009) have detailed extensive gene expression differences between mouse and human macrophages generated in vitro using each cytokine, and therefore there is some merit in considering that CSF-1- and GM-CSF-derived macrophages have functional distinctions. The two problems with this nomenclature are that (a) it is unclear what one would

call a CSF-1-derived macrophage stimulated with IFNγ vs IL-4 (e.g., M1–M1 vs M1–M2), and (b) it is confusing in terms of the other definitions.

In this review, we will correlate our terminology closely with the Ivashkiv definitions, but incorporate the additional criteria of the stimulatory cytokines and predictive gene expression signatures, as previously described. Therefore, we will refer to macrophages exposed primarily to T_H2 cytokines as M2. Macrophages stimulated with IFNγ in the presence of microbial stimulation will be referred to as M1, noting that this definition combines Gordon's "classical" activation groups.

24.2 Intrinsic and Extrinsic Pathways Regulate Macrophage Polarization

The phenomenon of macrophages adopting biased M1 or M2 phenotypes can occur in an apparent intrinsic way, or more commonly via extrinsic pathogenic or cytokine cues (e.g., activation of Toll-like receptors and IFNγ for M1-type macrophages and IL-4, IL-13 for M2-like macrophages). Examples of intrinsic signals have been recently reviewed by Ivashkiv and can involve chromatin and transcriptional changes that bias macrophage phenotypes (Ivashkiv 2012). Another example is the adaptor protein Tribbles 1 (*Trib1*), with deficiency in *Trib1* resulting in low numbers of M2 polarized tissue macrophages due to excessive expression of C/EBPα that inhibits the development of these cells (Satoh et al. 2013). Collectively, intrinsic signals that regulate polarization are an emerging field. We predict that many factors will be uncovered that bias the epigenetic landscape of developing macrophages such that gene expression linked to polarization states becomes enforced, regardless of the extrinsic signals received.

24.3 Cytokine Mediated STAT Signaling is Feedback Inhibited by SOCS Proteins

One of the primary determinates of macrophage polarization is exposure to extrinsic cytokine signals. Following binding to their cognate receptor complex, the type I and type II cytokines signal through the receptor-associated JAK/STAT pathway (Kiu and Nicholson 2012). Signaling is initiated by juxtaposition and activation of the JAK kinases, which in turn phosphorylate tyrosyl residues within the receptor cytoplasmic domains to recruit a second wave of SH2-domain containing signaling intermediates; prominent among them are the signal transducers and activators of transcription or STATs. The STATs are themselves phosphorylated on a critical C-terminal tyrosine residue, resulting in reorientation of the dimeric STAT unit and subsequent STAT translocation to the nucleus to initiate a complex series of transcriptional changes. The expression and/or repression of STAT-regulated genes

not only serves to polarize the macrophage phenotype but also results in the transcription of genes encoding a family of small, negative regulators, known as the SOCS proteins.

The canonical SOCS family is comprised of eight proteins (SOCS1-7 and CIS). Each family member was defined on the basis of sequence similarity and contains a "SOCS box" that couples the SOCS protein to a Cullin Ring E3 ligase complex, and an SH2 domain that binds phosphotyrosines on the SOCS target protein (Alexander and Hilton 2004). Many other proteins contain SOCS boxes coupled to alternate protein-interaction domains and some, such as the SPRY domain-containing SOCS box (SPSB) proteins, have key roles in regulating macrophage activation (discussed below) (Kuang et al. 2010). The N-termini of SOCS proteins vary considerably in length and sequence composition, with SOCS4-7 containing extensive N-terminal regions. SOCS1 and SOCS3 inhibit cytokine signaling through a unique kinase inhibitory region (KIR), which is required for inhibition of the JAKs (Babon et al. 2012; Kershaw et al. 2013; Sasaki et al. 1999). The structure and function of CIS and SOCS1-3 is now well established and has been extensively reviewed (Babon and Nicola 2012; Linossi et al. 2013). In essence, the expression of SOCS-encoding genes is induced in response to cytokine signaling. The SOCS then bind their target phosphotyrosine residues within cytokine receptor cytoplasmic domains and this enables SOCS1 and SOCS3 to bring the KIR in the vicinity of the receptor bound JAK: kinase inhibition ensues dampening the JAK-dependent output. SOCS3 (and most likely SOCS1) interacts with JAK1, JAK2, and TYK2 via a phosphorylation-independent interaction involving the KIR and SH2 domain which blocks substrate binding to the JAK JH1 domain (Kershaw et al. 2013). Mice lacking the SOCS1 or SOCS3 SOCS box have mild phenotypes compared to the lethal consequences of *Socs1* or *Socs3* deletion (discussed below) arguing that the KIR is more important than SOCS box-mediated ubiquitination and proteasomal degradation in extinguishing cytokine signaling (Boyle et al. 2009; Linossi et al. 2013; Zhang et al. 2001). This is consistent with the relatively weak affinity of the SOCS1 and 3 SOCS boxes for Cullin5, and further predicts that SOCS box-mediated E3 ligase activity will have a greater role in the function of the remaining SOCS family proteins (Babon et al. 2009). The specificity of the entire system remains an outstanding question, as while SOCS1-3 have defined substrates (and probably many unknown substrates), the substrate range of SOCS4-7 and CIS is unclear. In this chapter we will confine discussion of the canonical SOCS family to SOCS1-3 as these proteins are closely tied to macrophage polarization.

The ability of SOCS1 to regulate IFNγR signaling is a prototypic example of canonical SOCS function. Following IFNγ activation of JAK and STAT1, SOCS1 is induced and binds to a single phosphotyrosine within the IFNγR (Starr et al. 2009). The SOCS1 KIR then inhibits the receptor-associated JAK1 (and possibly JAK2, as IFNγR signaling requires both JAK1 and JAK2 (Murray 2007). SOCS1 is essential for inhibiting IFNγR signaling as shown in *Socs1*-deficient mice (SOCS1 KO) which manifest a lethal inflammatory response days after birth that can be rescued by independent deletion of *Ifng*, STAT1 or the elimination of lymphocytes

as a source of IFNγ (Alexander and Hilton 2004; Alexander et al. 1999). A variety of other manipulations have been performed to modify the *Socs1* knockout phenotype and these have further advanced knowledge of the range of SOCS1 substrates sub-dominant to the IFNγR (e.g., the IFNα/βR and IL-12R are also SOCS1 targets) (O'Shea and Murray 2008). Similarly, SOCS3 has critical roles in regulating the LIFR, LeptinR, G-CSFR and any receptor complex containing gp130 (e.g., IL-6R, IL-11R) while SOCS2 blocks growth hormone receptor signaling (Metcalf et al. 2000; Murray 2007; O'Shea and Murray 2008). SOCS proteins not only regulate the magnitude of signaling, but can also modify the type of STAT signaling generated by a given receptor. A clear example is the SOCS3 knockout, where IL-6R signaling in the absence of SOCS3 activates excessive and long-lived phosphorylated STAT3 (STAT3-P), ectopic STAT1-P and a branch of STAT3 signaling normally activated by the IL-10R (Croker et al. 2003; Lang et al. 2003; Yasukawa et al. 2003). These findings have been reviewed extensively and will be further expanded upon here.

24.4 Regulation of SOCS Expression in Macrophages Undergoing STAT-Mediated Polarization

1. SOCS1 and macrophage polarization. Given that SOCS proteins are feedback inhibitors of cytokine receptor signaling it should be of no surprise that they are induced following cytokine stimulation of macrophages. Dickensheets et al. initially characterized the pattern of SOCS1-3 mRNA expression following macrophage stimulation with IFNγ, IL-10, IL-4, or GM-CSF; a surprising pattern of expression was observed where SOCS1 mRNA was specific to IFNγ and IL-4 stimulation, SOCS2 was induced by IL-4 and more weakly by GM-CSF, and with the exception of IL-4, SOCS3 was strongly induced by all factors (Dickensheets et al. 2007). From these data, Dickensheets et al. established that in addition to its ability to negatively regulate the IFNγR, SOCS1 was an essential repressor of IL-4R signaling, (Dickensheets et al. 2007). Most significantly, IL-4-induced STAT6 phosphorylation and expression of Arg1, a canonical M2 marker following IL-4R signaling, was dramatically increased in *Socs1*$^{-/-}$macrophages, indicating that IL-4R signaling is repressed by SOCS1 (note that Arg1 expression is also found in M1 macrophages following STAT3 activation by IL-6, IL-10, or G-CSF and is thus not always an M2 "marker" (Qualls et al. 2010)). These observations are supported by genetic evidence whereby deletion of the IL-4/13-specific *Stat6* gene also ameliorated the inflammatory phenotype observed in *Socs1*-deficient mice (Naka et al. 2001). Since the earliest observed phenotype in *Socs1*$^{-/-}$ mice was hypersensitivity to IFNγ and excessive inducible nitric oxide synthase (iNOS) expression (a canonical marker of M1 macrophages) (Alexander et al. 1999), SOCS1 is a feedback inhibitor of *both* M1 and M2 macrophages.

Half a decade later, Whyte et al. attempted to link SOCS1 to macrophage polarization using siRNA-mediated "knockdown" approaches (Whyte et al. 2011). They found that with reduced *Socs1* levels, IL-4-induced Arg1 expression was decreased and this was linked to an increase in iNOS expression. Whyte et al. also found that Arg1 expression was increased after IFNγ and LPS stimulation (an observation where the authors failed to account for the link between the IL-6-STAT3 pathway and Arg1). Most troubling, Whyte et al. found that the amount of iNOS mRNA was *lower* in SOCS1 siRNA macrophages: the exact opposite of the original genetic experiments in the *Socs1*$^{-/-}$ mice that established an essential role for SOCS1 in suppressing IFNγ-induced iNOS expression (Alexander et al. 1999). Whyte et al. also failed to correlate STAT activation to the observed changes in downstream gene expression. Collectively, the Whyte study cannot be reconciled with the genetic-based studies without a side-by-side comparison of the two methodologies. Therefore, we suggest that further experiments using genetic models coupled to STAT-P measurements, and not siRNA, should be used to address the role of SOCS1 in polarization.

2. SOCS2 and macrophage polarization. *Socs2*$^{-/-}$ mice have gigantism caused by excessive GHR signaling (Metcalf et al. 2000). In macrophages, SOCS2 mRNA is strongly induced by IL-4R signaling (Dickensheets et al. 2007) and we might anticipate that the absence of SOCS2 could either cause increased IL-4R signaling and increased M2 polarization, or alternatively if it functions to cross-regulate M1 cytokines, increased M1 polarization. To date only one study has investigated "polarization" in the absence of SOCS2: these authors found that *Socs2*$^{-/-}$ mice have greatly increased susceptibility to LPS-induced shock coupled with increased production of IL-6, TNF and IFNγ and decreased expression of some M2 markers (Spence et al. 2013). These data are difficult to interpret for a number of reasons. First, the authors did not establish the target of SOCS2 responsible for the decreased M2 phenotype; this may be any one of a number of cytokine receptors linked to extrinsic polarization. Second, Spence et al. failed to examine the in vivo effects of *Socs2* deletion on M2 polarized responses such as worm infections or allergy and asthma models, it would be important to tie the known expression pattern of SOCS2 mRNA with polarization phenotypes.
3. SOCS3 and macrophage polarization. In macrophages SOCS3 primarily regulates gp130 signaling (Murray 2007). When SOCS3 is absent several signaling perturbations occur; there is elevated and long-lived STAT3-P after IL-6 stimulation and this is coupled with increased STAT1-P (Croker et al. 2003; Lang et al. 2003). The increased STAT3-P "signal" results in ectopic activation of an IL-10-like STAT3 anti-inflammatory response (El Kasmi et al. 2006; Yasukawa et al. 2003). In terms of polarization, a further facet is that the *Il4ra* gene (encoding the IL-4Rα) is a target of STAT3, and especially STAT3 activated from the IL-10R (Lang et al. 2002). Increased IL-4Rα expression leads to increased responsiveness to IL-4 and IL-13, and potentially to increased M2 polarization. Finally, the STAT3 pathway is a potent activator of Arg1 expression (in addition to Arg1 expression driven by the "canonical" M2 STAT6-dependent pathway) and indeed, autocrine production of IL-6 increases Arg1 mRNA and protein in the

absence of SOCS3 (El Kasmi et al. 2008; Qualls et al. 2010). Based on these studies we can hypothesize that deletion of *Socs3* might result in reduced M1 activity, coupled with increased IL-4Rα and Arg1 expression and the potential for elevated M2 polarization. With this background in place, we can now discuss the outcomes from three studies that set out to test how SOCS3 regulates polarization.

Liu et al. used siRNAs to deplete SOCS3 mRNA and found increased Arg1 and what they claimed were increased anti-inflammatory effects (Liu et al. 2008). Similarly, Spence et al. found that mice bearing conditional alleles of *Socs3* (crossed to the LysM-Cre to delete in myeloid cells) survive LPS challenge due to ectopic activation of the IL-10 signaling pathway, also previously shown by Yasukawa et al. (Spence et al. 2013; Yasukawa et al. 2003). Spence et al. also showed that SOCS3-deficient macrophages express increased M2 markers (*Arg1*, *Chi3l3*, *Retnla*) as would be expected for cells that have increased IL-4/IL-13 signaling stemming from increased cell surface amounts of IL-4Rα: a fact neglected by the authors. It is also important to note that under some circumstances macrophages can produce IL-4 and IL-13 that can then signal in an autocrine-paracrine way to further enhance M2 polarization (Qualls et al. 2012; Shirey et al. 2008, 2010).

Similarly to Spence et al., Benveniste and colleagues also investigated the effects of conditionally deleting the *Socs3* on polarization in two studies and came to a diametrically opposite conclusion from the other groups noted above (Qin et al. 2012a, b). Qin et al. observed increased iNOS and IL-23p19 mRNA expression (denoted as useful markers of M1 polarization (Murray and Wynn 2011b), decreased IL-10, and *increased lethality* after LPS challenge, with the caveat that the challenge models were distinct between Qin et al. and the Yasukawa et al. paper. When considered together, the effects of SOCS3 on polarization require significant re-evaluation.

24.5 Interpretation and Predictions About the Effects of SOCS1-3 Mutations

A major drawback of interpreting research on the connections between SOCS1-3 and polarization is that different groups use very different cell populations, assay systems and readouts to evaluate their data. We suggest that it would be more logical to assess the polarization responses of different macrophages from each knockout in a head-to-head way. Certain caveats are necessary because of the lethality of mice lacking *Socs1* and *Socs3* compared to *Socs2* (a problem encountered by Spence et al. who compared the complete knockout of *Socs2* to the conditional allele of *Socs3*) and because the targets of SOCS2 have not been identified. An ideal approach would be to use conditional alleles of all mice with a matched Cre deleter. Based on our existing understanding of SOCS regulation of cytokine signaling, predictable outcomes can be made and are diagrammed in Fig. 24.1.

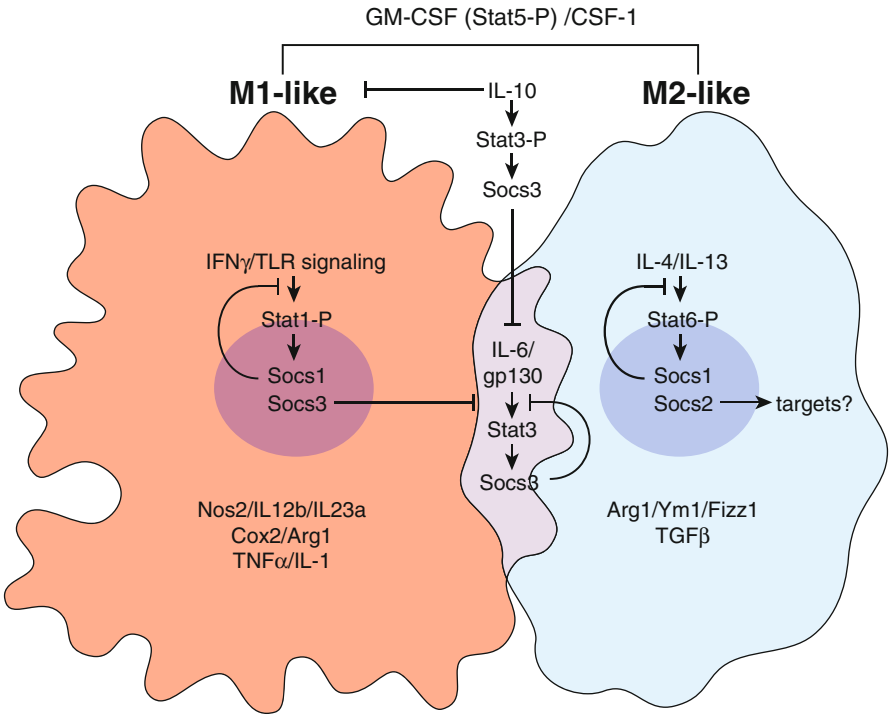

Fig. 24.1 Simplified overview of the potential effects of the STAT/SOCS pathway in macrophage polarization. In *orange* are M1-like cells and in *blue* are M2-like cells that arise under the overlapping influences of GM-CSF and CSF-1. In M1 cells, IFNg/TLR signaling (indirectly via type I IFNs) activates SOCS1 and SOCS3 to negatively regulate IFNR (Type I and II) and IL-6R signaling. In M2 cells, SOCS1 and SOCS2 are activated. SOCS1 blocks IL-4R signaling while the target of SOCS2 remains unknown. SOCS3 has a common effect in blocking gp130 mediated signaling regardless of the macrophage type. SOCS3 is also strongly induced by IL-10, whose anti-inflammatory effects are pan-macrophage. Note that IL-10R signaling is not blocked by SOCS3 or any SOCS protein as the IL-10R lacks SOCS binding sites. Typical marker genes for M1 and M2 macrophages are shown; noting that Arg1 is highly expressed in both M1 and M2 macrophages, but by different cytokine signaling mechanisms

24.6 SOCS-Box Protein Modulation of Polarization Effects

As mentioned, the SPSBs are another SOCS box family that has emerged as critical regulators of, in particular, M1 macrophage effector function. SPSB1, 2, and 4 contain an SPRY-interaction domain which interacts with an "NNN" motif in iNOS to down-regulate iNOS expression via the SOCS box-associated E3 ligase (Kuang et al. 2010; Nishiya et al. 2011). Interestingly, SPSB1 expression is regulated in BMDM by TGFβ so in theory, the production of TGFβ by M2 macrophages may result in dampening of M1 effector responses via SPSB1 ubiquitination of

iNOS. This complex interplay does highlight the potential role that ubiquitination per se is likely to have in regulating macrophage polarization and function, and we predict that future studies will identify many SOCS box and SOCS-independent examples of ubiquitin-mediated modulation of the critical signaling pathways.

24.7 Conclusions

While it is clear that SOCS proteins will have important roles in regulating macrophage polarization, the studies to date have perhaps only highlighted the complexities of STAT–SOCS regulation, and further more definitive investigations are needed, firstly to address the in vivo impact of SOCS-deletion on macrophage polarization and secondly, to define the biochemical targets of individual SOCS proteins in M1 vs M2 macrophages.

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Chapter 25
Functions of the Large Maf Transcription Factors and Macrophages

Michito Hamada and Satoru Takahashi

25.1 Introduction: What Are Maf Transcription Factors?

Maf transcription factors are a group of transcription factors identified as homologs of v-Maf, which was first discovered from the AS42 virus that causes musculoaponeurotic fibrosarcoma in chicken (Nishizawa et al. 1989). These transcription factors bind to a DNA sequence called Maf recognition element (MARE) through a bZip structure, which comprises of a leucine zipper structure required for dimer formation as well as a basic domain involved in DNA recognition. The Maf transcription factors are divided into two subfamilies: one subfamily is called the large Maf transcription factors, each member of which contains a bZip structure and a transcription activating domain, and the other subfamily is called the small Maf transcription factors, each member of which contains only the bZip structure (Motohashi 1997; Blank and Andrews 1997). In humans and mice, four large Maf transcription factor encoding genes [namely, *c-Maf* (a cellular counterpart of *v-Maf*), *MafA*, *MafB,* and *Nrl*] and three small Maf transcription factor encoding genes (namely, *MafK*, *MafG,* and *MafF*) have been identified so far (Fig. 25.1). As the small Maf transcription factors do not possess any transcriptional activation domain, they function either as a transcription inhibitor by forming a homodimer of two small Maf transcription factors or as a transcription activator by forming a heterodimer with an activation domain-containing transcription factor (Igarashi et al. 1994). The large Maf transcription factors also

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Fig. 25.1 Maf transcription factor family. There are four large Maf and three small Maf transcription factors in both human and mouse. Large Mafs have transcriptional activation domain in the N terminus, on the other hand, small Mafs do not. In the large Mafs, they are c-Maf, MafB, MafA, and Nrl, respectively. Usually, large Mafs form homodimer and bind to the target DNA sequences Maf recognition element (MARE) and activate gene transcription

form Maf homodimers as well as Maf heterodimers with other compatible bZIP proteins. Analyses of knockout mice lacking these Maf transcription factor genes revealed that the large Maf transcription factors are expressed in various tissues and cells, such as the nerves (Blanchi et al. 2003), crystalline lens of the eye (Bessant et al. 1999a), retina (Kawauchi et al. 1999), and pancreas (Olbrot et al. 2002), as well as in the hematopoietic cells including macrophages (Kelly et al. 2000a) and T cells (Ho et al. 1996), and that the large Maf transcription factors play critical roles in the development and function of these cells (Table 25.1). Ectopic expression of the large Maf transcription factors, however, could lead to cancer development. Accordingly, in human multiple myeloma cases (Chesi et al. 1999) and in mouse models (Morito et al. 2006, 2011) cancerous changes in cells were simply induced by the overexpression of the large Maf proteins, a result that is different from the other types of cancers caused by transcription factors, where chimeric proteins are formed.

In this manuscript, we have reviewed the functions of large Maf transcription factors in macrophages.

Table 25.1 Functions of large Maf transcription factors in cells/tissues other than macrophages and related human diseases

Large Mafs	In vivo function	Related human diseases
c-Maf	Lens fiber cell development Kawauchi et al. (1999) Sensory neuron development Wende et al. (2012) Transcription of IL-4 in T cells Ho et al. (1996) Osteoblast development Nishikawa et al. (2010)	Cataract, Iris coloboma Jamieson et al. (2002) Abnormality of Pacinian corpuscle Wende et al. (2012) Multiple myeloma Chesi et al. (1999) Angioimmunoblastic T cell lymphoma (AITL) Murakami et al. (2007)
MafB	Rhombomere segmentation Cordes and Barsh (1994) Podocyte development Moriguchi et al. (2006) Osteoclast development Kim et al. (2007) α and β cell development Artner et al. (2007)	Nephrosis Zankl et al. (2012) Multicentric carpotarsal osteolysis Zankl et al. (2012) Multiple myeloma Chesi et al. (1999)
MafA	Insulin transcription Olbrot et al. (2002)	Diabetes Noso et al. (2010) Multiple myeloma Chesi et al. (1999)
Nrl	Rod cell development Bessant et al. (1999b)	Retinitis pigmentosa Bessant et al. (1999b)

25.2 Expression of Large Maf Transcription Factors in Macrophages

Large Maf transcription factors were shown to be expressed in a variety of cells (Kajihara et al. 2001). For example, it was reported that MafB is expressed in hematopoietic stem cells, through which it negatively controls the differentiation of macrophage cells in the hematopoietic system by regulating the signaling of M-CSFR (Sarrazin et al. 2009). Gene expression-array analyses of various subset genes of dendritic cells, macrophages, and other cells revealed that c-Maf and MafB are expressed strongly in the red pulp macrophages, but hardly in the alveolar macrophages (Gautier et al. 2012; Satpathy et al. 2012). MafB is also expressed in macrophage dendritic cell progenitor (MDP), thus suggesting that MafB is involved in deciding the differentiation fates of the macrophages and dendritic cells (Satpathy et al. 2012; Bakri et al. 2005). Almost no expression of the other two large Maf transcription factors—namely, MafA and Nrl—was observed in the monocyte–macrophage lineage cells (Fig. 25.2).

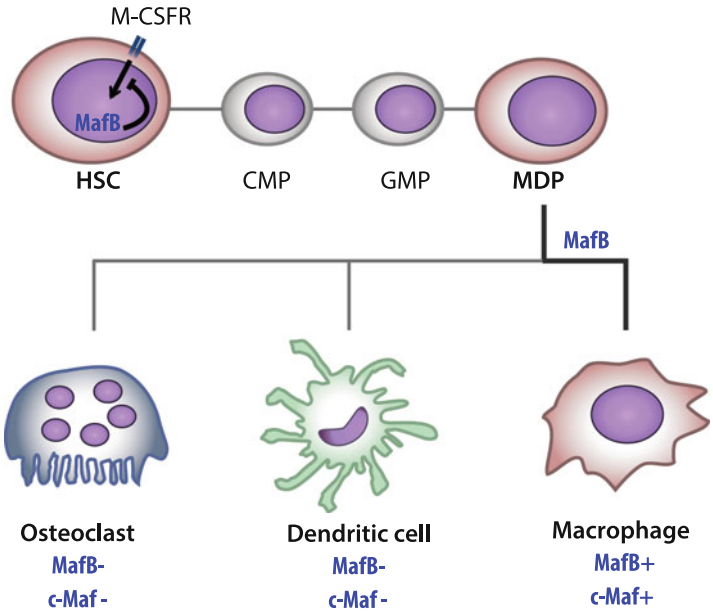

Fig. 25.2 Expression of c-Maf and MafB in monocyte–macrophage lineages. MafB inhibits M-CSF signals in HSC (Sarrazin et al. 2009). In MDP, MafB may promote macrophage differentiation (Satpathy et al. 2012). *HSC* hematopoietic stem cell, *CMP* common myeloid progenitor, *GMP* granulocyte macrophage progenitor, *MDP* macrophage dendritic cell progenitor

25.3 Functions of Large Maf Transcription Factors in Macrophages

25.3.1 Role of MafB in the Development of Macrophages

In 1990s, Thomas Graf et al. demonstrated using chicken cells (Kelly et al. 2000b) and cell lines (Sieweke et al. 1996) that MafB stimulates the differentiation of macrophages. Recent studies, on the other hand, showed that a reduction in the *Mafb* expression promoted differentiation of progenitor cells into dendritic cells and osteoblasts, both of which belong to the monocyte–macrophage lineage (Bakri et al. 2005; Kim et al. 2007). Nevertheless, no significant abnormalities were observed in MafB deficient mouse; in this study, however, detailed analysis focussing on dendritic cells and osteoclasts was not performed (Moriguchi et al. 2006). Further studies are therefore needed for delineating the role of MafB. Recently, genomic sequence analysis in patients with multicentric carpotarsal osteolysis, in which osteolysis and kidney impairment are exacerbated, revealed that all 11 test cases carried mutations in the transcriptional activation domain of *MAFB* (Zankl et al. 2012), suggesting that in human too MafB inhibits the differentiation of osteoclasts and promotes the

differentiation of macrophages. Together, these results suggested that MafB inhibits the differentiation of cells that are closely related to macrophages, such as dendritic cells and osteoclasts, and regulates the differentiation of macrophages.

25.3.2 Functions of MafB in Macrophages

It was shown that the *Mafb*-deficient macrophages exhibited increased responsiveness to M-CSF and formed more colonies (Sarrazin et al. 2009). In addition, it was shown that the expression of F4/80, which is an immunosuppressive receptor expressed on the cell surface of macrophages, was decreased. These results are consistent with the fact that the MARE sequence is conserved in the regulatory region of the *F4/80* gene, and the expression of *F4/80* is positively regulated through direct binding of MafB to this element (Moriguchi et al. 2006).

Early studies have suggested no abnormalities in the phagocytic capacity of *Mafb*-deficient macrophages against nonspecific foreign substances, such as latex beads (Moriguchi et al. 2006). Subsequently, our detailed study revealed decreased production of C1q in the complement system of the *Mafb*-deficient macrophages, and also showed a significant decrease in the phagocytic capacity of apoptotic cells. Among the molecules of the complement system, C1qa, C1qb, and C1qc are mainly produced in macrophages, and it has been demonstrated that these molecules are used for the elimination of apoptotic cells produced in the body by activating the classical pathway of the complement system. In fact, *C1qa*-deficient mice showed abnormality in eliminating the apoptotic cells and exhibited autoimmune disease-like phenotypes (Botto et al. 1998). It is also known that 90 % of genetically *C1q-deficient* human patients develop SLE (Petry et al. 1997). Therefore, regulation of C1q production is very important for eliminating apoptotic cells from the body. It was shown that the regulatory regions of *C1qa*, *C1qb,* and *C1qc* genes carried MAREs, and these genes were direct targets of MafB. Thus, as the abovementioned results suggest, MafB is an important regulatory molecule for the phagocytosis in macrophages, and it acts through the complement system.

When macrophages phagocytose foreign substances, deaths of these macrophages needed to be suppressed to keep these foreign substances inside the cells. Accordingly, an apoptosis inhibitor of macrophage (AIM) was identified as a molecule playing such a role (Arai et al. 2005). AIM is a secretory protein whose expression in the macrophage is induced by LXRα, and it suppresses apoptosis of macrophages by affecting the macrophage per se (Arai et al. 2005). It was shown that MafB is essential for the induction of AIM (Arai et al. 2005). Consistent with the above observation it was shown that in *Mafb*-deficient macrophages, cells that phagocytosed oxidized lipid had undergone apoptosis because AIM was not induced (Hamada et al. 2014).

Thus, as discussed above, the most important functions of MafB are to control some of the vital functions of macrophages, such as engulfing apoptotic cells and foreign substances and keeping them inside the cells (Table 25.2).

Table 25.2 Functions of MafB in macrophages

Function of MafB	Details of function
Stimulate differentiation of macrophage	Stimulates macrophage differentiation form hematopoietic stem cells and suppresses osteoclast differentiation Bakri et al. (2005), Kim et al. (2007)
Regulation of signaling from M-CSF receptor	Negatively regulates growth signal from M-CSF receptor Sarrazin et al. (2009)
Suppression of apoptosis	Induces apoptosis inhibitor of macrophage (AIM) Arai et al. (2005)
Stimulation of phagocytosis	Induces C1q expression and enhances phagocytosis of apoptotic cells (our unpublished observation)
Upregulation of F4/80	Induces expression of F4/80, which is one of the immunosuppressive molecules on macrophages Moriguchi et al. (2006)

25.3.3 Functions of c-Maf in Macrophages

Hypoplastic defect of the lens and dysosteogenesis were observed in *c-Maf-deficient* mice; it was, however, shown that, on a C57BL/6 genetic background, the *c-Maf-deficient* mice died in the late fetal period due to anemia (Kusakabe et al. 2011). Further analysis suggested that the embryonic anemia was not due to any abnormality in the erythroid cells themselves, but was due to the malformation of erythroblastic islands, which mainly consist of macrophages and are formed in the fetal liver. In vitro erythroblastic island reconstitution experiments using *c-Maf*-deficient macrophages demonstrated that adhesion of macrophages with erythroblasts was decreased, causing insufficient formation of erythroblastic island (Kusakabe et al. 2011). Expression of VCAM-1 was found to be decreased in the *c-Maf*-deficient macrophages. Since the regulatory region of *VCAM-1* gene contained MAREs, it was therefore suggested that the decrease in VCAM-1 expression might be the underlying cause for the failure of erythroblastic island formation (Kusakabe et al. 2011).

c-Maf has been shown to be an essential transcription factor for the production of IL-4 in helper T cells. c-Maf is also shown to be essential for the production of IL-10 in macrophages. Previous studies have shown that the cytokine IL-10 suppresses immune response, and immune-mediated enteritis in mouse is known to be caused by a deficiency of IL-10 (Cao et al. 2002, 2005). It is also known that c-Maf controls the expression of F4/80, which is important for the induction of immune tolerance (Nakamura et al. 2009). Therefore, c-Maf is an important transcription factor involved in the regulation of immunosuppressive function of macrophages.

It is also known that macrophages deficient in both *MafB* and *c-Maf* lose their ability to inhibit proliferation, and they undergo self-renewal. Hence, it has been suggested that MafB and c-Maf may have overlapping functions (Aziz et al. 2009) (Table 25.3).

Table 25.3 Functions of c-Maf in macrophages

Function of c-Maf	Details of function
Erythroblastic island formation in embryonic stage	Induces VCAM-1 expression and stimulates formation of blood island Kusakabe et al. (2011)
IL-10 expression	Induces IL-10 transcription though MARE site in IL-10 gene regulatory region Cao et al. (2002, 2005)
Upregulation of F4/80	Induces expression of F4/80, which is one of the immunosuppressive molecules on macrophages Nakamura et al. (2009)

25.4 Conclusion

Among the large Maf transcription factors, MafB and c-Maf are expressed in macrophages except in alveolar macrophage, and they are essential for the regulation of fundamental functions of macrophages, such as phagocytosis of dead cells and lipids, as well as highly sophisticated functions, such as erythroblastic island formation and cytokine production. Considering the facts that MafB inhibits the differentiation of progenitors into other lineages, such as dendritic cells and osteoclasts, and controls macrophage differentiation from the hematopoietic stem cells, and that both MafB and c-Maf inhibit the self-renewal of macrophages, it can be concluded that MafB and c-Maf control various aspects of microphage population.

In each tissue, macrophages exist from the early stages of development and remain alive for maintaining a certain ratio until the death of the individual multicellular organism. The large Maf transcription factors MafB and c-Maf, which control macrophage function, differentiation, and number, are thus needed for the survival of macrophages in the tissue.

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Chapter 26
The Control of Gene Expression in Macrophages

Renato Ostuni and Gioacchino Natoli

26.1 Introduction

Macrophages play crucial biological functions, both at the steady-state and in response to micro-environmental stimulation, notably danger signals. At steady-state, macrophage properties are generally controlled by local concentrations of cytokines that instruct trophic and housekeeping functions of macrophages in a tissue-specific fashion (Wynn et al. 2013). These responses represent intrinsic features of the developmental programs of macrophages. Inducible responses of macrophages are instead triggered by the acute or chronic detection of subtle micro-environmental changes through a wide panel of dedicated receptors (Medzhitov 2008). Macrophage activation is finely tuned by specific and complex environmental contexts, and therefore can be interpreted as a continuum of states comprised between two extreme phenotypes: classical (or M1) and alternative (M2) activation. Classically activated, M1-polarized macrophages are typically induced by the T helper 1 (T_H1)-promoting cytokine interferon-γ (IFNγ) and display a transcriptional program characterized by the elevated expression of pro-inflammatory genes such as *Nos2*, *Il12b,* and *Ciita*. Similar, although not identical, gene expression programs can also be triggered by the recognition of pathogen-associated molecular patterns (PAMPs) through a set of pattern recognition receptors (PRRs), as exemplified by the Toll-like receptor 4 (TLR4)-dependent activation of macrophages in response to stimulation with lipopolysaccharide (LPS). Alternative activation (or M2 polarization) of macrophages is induced by the T_H2-promoting cytokines interleukin-4 (IL-4) and IL-13 and results in the up-regulation of genes such as *Arg1*, *Retnla*, *Chi3l3*, *Mrc1*, which are involved in the resolution of inflammation, tissue repair, and wound healing (Lawrence and Natoli 2011).

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Regardless of the nature of the stimulus, a general feature of macrophage responses to environmental changes is a highly regulated reorganization of their gene expression program, which involves the quantitative, qualitative, and temporal regulation of genes that in turn specify a precisely tailored biological response.

26.2 Context-Dependent Gene Expression: Achieving Stimulus-Specific and Cell-Type Specific Responses

An emerging general concept in transcriptional regulation is that *transcriptional programs integrate two types of information: cell identity and environmental stimulation* (Natoli 2010). The precise identity of a cell is typically defined during the course of alternative differentiation pathways, driven by specific combinations of transcription factors (TFs) acting in lineage determination and induced by specific stimuli (Mossadegh-Keller et al. 2013) or activated as a result of stochastic decisions (Naik et al. 2013). However, since in complex organisms cells are not isolated from the environment, and in fact are heavily influenced by it, their transcriptional programs are finely tuned and reshaped by the action of TFs induced by stimulation. The integration of both types of information is responsible for the generation of *context-dependent gene expression programs*, meaning that (1) *a given cell type responds differently to different stimuli* (*stimulus-specificity*) *and* (2) *different cell types respond differently to the same stimulus* (*cell-specificity*). Understanding the principles underlying these mechanisms of transcriptional regulation is of paramount biological importance, especially in the context of macrophages, in which the regulated gene expression is crucial for their biological functions, and loss of this control often results in disease (Smale 2010). Synergistic activation of the proper combinations of TFs in response to stimulation is crucial to achieve stimulus-specific gene induction; however, additional layers of regulation exist that reflect the fact that *accessibility of the DNA is intrinsically controlled by its organization into chromatin*. These aspects and the ensuing models of transcriptional control are discussed below.

26.2.1 Chromatin Determinants of Stimulus-Specific Gene Induction in Macrophages

26.2.1.1 General Principles: The Functional Organization of Mammalian Genomes

The fundamental unit of chromatin is the nucleosome, which consists of approximately 147 base pairs (bp) of DNA wrapped around an octameric complex of the core histones H3, H4, H2A, H2B. This spatial organization is crucial to solve a dimensional problem, i.e. to fit linear DNA molecules into a 1–10 μm-sized nucleus

in eukaryotic cells, and at the same time provides multiple regulatory layers impacting on gene expression. The ensemble of chromatin modifications that characterize a given cell type at a given time is collectively defined the *epigenome* of that cell.

DNA Modifications

The primary layer of transcriptional regulation resides in the DNA sequence itself, which specifies both its coding and regulatory potential by acting as template for mRNA transcription and by providing specific binding sites for sequence-specific TFs. DNA sequence is affected by chemical modifications, like the global methylation of cytosines (meC) in the context of CpG dinucleotides (Deaton and Bird 2011). Because of the mutagenic properties of meC, the genome of vertebrates is globally CpG-depleted; however, this landscape is punctuated with unmethylated, CpG-rich regions termed *CpG islands*. These elements are positively associated with gene expression: around 70 % of annotated promoters coincide with CpG islands, and a large fraction of their target genes (mostly housekeeping, and partly environmental genes) are actively transcribed. Notably, CpG islands are not exclusively found near transcription start sites (TSSs) of annotated protein-coding genes, as a significant fraction of them localize in gene-distal regions, largely coinciding with extragenic sites of transcription initiation that in some cases correspond to non-protein-coding genes (Wiench et al. 2011).

Nucleosome Organization

The organization of DNA into nucleosomes exerts both structural and regulatory functions: it allows genome compaction and stabilization, and contributes to transcriptional regulation through histone variants and histone post-translational modifications (see below). Furthermore, the positioning of nucleosomes along the genome directly impacts on the accessibility of the underlying DNA to *trans*-acting factors. Intuitively, TFs can only access DNA regions that are either nucleosome-depleted or in which nucleosome positioning is not stably fixed. Notably, both conditions can be subjected to dynamic alterations by the activity of chromatin remodeling complexes belonging to the SWI/SNF, INO80, ISWI, and CHD families, which use ATP to actively evict or slide nucleosomes (Hargreaves and Crabtree 2011). This indicates that inducible gene expression is not only dictated by the basal nucleosomal organization at regulatory elements such as promoters and enhancers, but also depends on how this organization is dynamically modified after stimulation.

Histone Modifications

Nucleosomal histones are globular proteins except for an unstructured N-terminal tail, which undergoes an impressive combination of many post-translational modifications. We refer the reader to excellent reviews describing the spectrum of known

histone modifications, how these are deposited, and how they are translated into function (Bannister and Kouzarides 2011; Suganuma and Workman 2011). We will focus our discussion on how different histone modifications and other chromatin features are specifically associated to *cis*-regulatory elements, providing a convenient way to functionally annotate the mammalian genome.

Promoters are typically defined by their proximal locations relative to the TSS of a gene (±2 kb), a feature that has greatly facilitated their genomic annotation. Features of active chromatin are globally observed at promoters of transcribed genes: these include tri-methylation on lysine 4 of histone H3 (H3K4me3), histone acetylation, replacement of canonical histones with the histone variants H2A.Z or H3.3, and hypersensitivity to DNase I (Barski et al. 2007; Heintzman et al. 2007; Wang et al. 2008; Thurman et al. 2012). Additionally, most constitutively active promoters coincide with unmethylated CpG islands. These features collectively create a permissive environment for TF binding and transcriptional competence. On the other hand, promoters of inactive genes are often enriched in repressive marks such as H3K27me3 and H3K9me3, they lack histone acetylation and are relatively resistant to DNase I digestion. Such chromatin configuration substantially impairs the usage of these regulatory elements by preventing TF binding.

Enhancers are the most common class of genomic *cis*-regulatory elements. They are located over a wide range of distances (up to about one megabase) away from their target gene, a feature that hampered their genome-wide annotation (Bulger and Groudine 2011). In recent years, pioneering studies provided the first indications that distal enhancer regions were associated with a rather specific chromatin signature involving high levels of H3K4me1 and low levels of H3K4me3, the opposite being observed at active promoters (Heintzman et al. 2007; Consortium et al. 2007). The presence of additional histone modifications such as H3K27ac and H3K9ac (Creyghton et al. 2010; Ernst et al. 2011; Bonn et al. 2012), the unstable histone variants H3.3 and H2A.Z (He et al. 2010; Jin et al. 2009) indicate transcriptional functionality, whereas enrichment in H3K27me3 at these regions is associated with a developmentally repressed state (Rada-Iglesias et al. 2011).

26.2.1.2 Chromatin Dynamics at Inflammatory Genes

Macrophage activation by inflammatory stimuli triggers the activation of TFs of multiple families, including nuclear factor-κB (NF-κB), activating protein-1 (AP-1), and interferon regulatory factors (IRFs) (Takeuchi and Akira 2010). The combinatorial activation of these TFs in turn defines a highly specific transcriptional output featuring the expression of direct and indirect effectors acting during distinct phases of the response. Tight control of the inflammatory gene expression program entails several regulatory mechanisms, including the temporally coordinated induction of hundreds of genes (Amit et al. 2009) and the control of mRNA stability and degradation (Rabani et al. 2011). Moreover, precise control of gene expression in response to inflammatory stimuli is regulated by chromatin features, some of which are already specified prior to macrophage activation (Smale 2010).

According to their induction kinetics, inflammatory genes can be broadly categorized into primary and secondary response genes (PRGs and SRGs) (Fowler et al. 2011). PRGs are rapidly induced after stimulation with no requirement for de novo protein synthesis. Therefore, they are direct targets of stimulus-activated TFs, as exemplified by the NF-κB-dependent induction of *Tnf*. SRGs are induced with much delayed kinetics (several hours post treatment) as the result of positive transcriptional feedbacks involving newly synthesized proteins, notably TFs. In the LPS response, a prototypical example is given by the interferon-responsive genes *Nos2* and *Ciita*; their transcription requires a first wave of interferon-β (IFNβ) synthesis, in turn promoting autocrine activation of STAT1/STAT2/IRF9 complexes (Lawrence and Natoli 2011).

The different induction kinetics of LPS-responsive genes to some extent reflects distinct chromatin organization modes at their promoters (Fig. 26.1). Early studies showed that, despite a quick and rather homogenous NF-κB nuclear translocation in response to LPS (Hoffmann et al. 2002), binding of NF-κB to the promoters of late genes was temporally delayed (Saccani et al. 2001). Based on these findings, it was

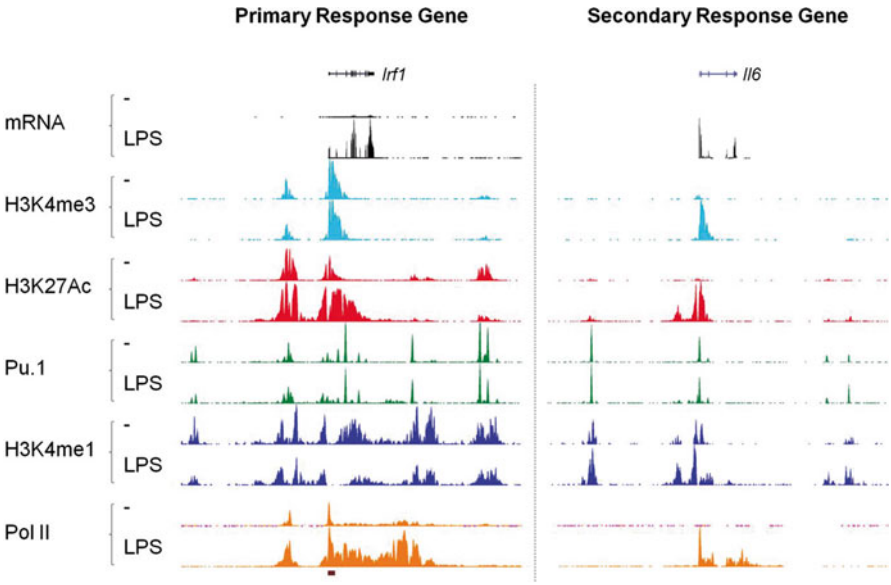

Fig. 26.1 Chromatin organization at primary and secondary response genes. A representative snapshot of chromatin organization at *Irf1* and *Il6* gene loci in untreated (–) and LPS-stimulated macrophages is shown (Ostuni et al. 2013). Consistent with an early induction kinetics, the CpG island-containing (*dark magenta box*) *Irf1* promoter is already enriched in the active chromatin marks H3K4me3 and H3K27Ac before stimulation; basal Pol II occupancy at the TSS can also be observed. After LPS treatment histone acetylation is further increased, and Pol II extends throughout the gene body, resulting in *Irf1* mRNA induction. Conversely, the secondary response gene *Il6* has an inactive promoter conformation, with almost undetectable levels of H3K4me3, H3K27Ac, and Pol II in unstimulated macrophages. H3K4me1 and Pu.1 peaks indicate enhancers, and are relatively stable (see text) (color figure online)

proposed that early genes had accessible promoters permissive for immediate NF-κB occupancy, whereas this was prevented in late genes by the presence of a nucleosome barrier (Natoli et al. 2005). Subsequent studies indeed showed that the LPS-mediated induction of SRGs (and some PRGs induced with late kinetics) was selectively abrogated by the knockdown of Brg1 and Brm, the catalytic subunits of the SWI/SNF chromatin remodeling complexes (Ramirez-Carrozzi et al. 2006). Notably, early genes whose induction did not require nucleosome remodeling were found to associate with promoters that were already accessible by nucleases in the basal state, whereas the promoters of late genes acquired a permissive state only after LPS treatment (Ramirez-Carrozzi et al. 2006; Saccani et al. 2001, 2002). A model for how this differential nucleosome organization at inflammatory gene promoters translates into specific patterns of gene induction was provided by the observation that promoters of most SWI/SNF-independent PRGs were enriched for CpG islands, whereas a strong association was not observed for SWI/SNF-dependent SRGs (Ramirez-Carrozzi et al. 2009; Escoubet-Lozach et al. 2011). Importantly, the mere presence of a CpG island intrinsically interfered with the assembly of stable nucleosomes at PRG promoters, as measured by in vitro assays (Ramirez-Carrozzi et al. 2009), and in vivo genome-wide studies (Valouev et al. 2011).

In addition to the global nucleosome depletion, CpG island promoters displayed relatively high levels of H3K4me3 and acetylated histones already in unstimulated macrophages (Fig. 26.1). Upon inflammatory stimulation, a substantial increase in H3K4me3 and H4K5/8/12Ac enrichment was observed at early gene promoters (De Santa et al. 2009; Foster et al. 2007; Ramirez-Carrozzi et al. 2009; Hargreaves et al. 2009; Austenaa et al. 2012), in agreement with the observed recruitment of p300/CBP histone acetyltransferase (HAT) at these sites (Ghisletti et al. 2010). Interestingly, H3K9/K14Ac remained substantially unchanged, suggesting different roles in transcriptional regulation for these histone marks (Escoubet-Lozach et al. 2011). Consistent with their permissive chromatin configuration, early gene promoters were found to be constitutively occupied by RNA Polymerase II (Pol II), mainly localized adjacent to the TSS-proximal +1 nucleosome (Adelman et al. 2009; Hargreaves et al. 2009; Escoubet-Lozach et al. 2011). This was reported to result in a basal, low-level transcription of these genes, generating long and unstable transcripts that could not be spliced into mature mRNA as a consequence of the lack of Pol II phosphorylation of serine 2 (Hargreaves et al. 2009), a critical Pol II elongation signal (Sims et al. 2004). Treatment of macrophages with LPS caused a massive increase in Poll II occupancy both at promoters and throughout gene bodies, thus indicating a switch from an initiating, unproductive Pol II to an elongating and fully competent state yielding mature mRNAs (Escoubet-Lozach et al. 2011; Hargreaves et al. 2009). Indeed, S2 phosphorylation of Pol II, which occurs upon promoter clearance and entry in the elongation phase, was only achieved upon LPS stimulation (Hargreaves et al. 2009).

Collectively, these findings identify a simple sequence feature, namely the presence of a CpG island, as the key element specifying the permissive chromatin state at promoters of most primary gene response, in turn facilitating their rapid induction upon stimulation. These features correlate with, and possibly influence, (1) the

deposition of histone marks positively associated with gene expression and (2) a basal level of abortive transcription that rapidly becomes productive upon microbial stimulation. However, a recent genome-scale analysis of nascent transcription in LPS-treated macrophages failed to confirm the dominant role of transcriptional elongation, rather than initiation, in induction of PRGs (Bhatt et al. 2012). No significant differences in the basal levels of transcription were observed between CpG-rich and CpG-poor genes, indicating that the permissive chromatin state at CpG-rich promoters does not necessarily imply basal transcriptional activity, possibly because of the lack in the basal state of the TFs required for transcription of some CpG-rich promoters (Bhatt et al. 2012).

A completely different situation is observed at late genes, whose promoters are basally inaccessible and not permissive for transcription, harboring low basal levels of H3K4me3 and H3/H4Ac (Escoubet-Lozach et al. 2011) (Fig. 26.1). Conversely, histone modifications associated with transcriptional repression may contribute to prevent leaky expression of both primary and SRGs. H3K27me3 was found to be enriched at a subset of inflammatory gene promoters (De Santa et al. 2009), and repression was relieved upon LPS stimulation at least partially through the histone demethylases JMJD3 and UTX (De Santa et al. 2009; Kruidenier et al. 2012); furthermore, H3K27me3 removal by JMJD3 was required to license *Irf4* expression and M2 polarization in mice challenged with chitin or helmints (Satoh et al. 2010). Basal enrichment of H3K9me2, deposited by the G9a/GLP methyltransferase complex, was observed at *Ifnb* and interferon-inducible genes in dendritic cells and macrophages and at even higher levels in fibroblasts (Fang et al. 2012). Finally, H3K9me3 and H4K20me3 were found to restrict TLR4 target genes in macrophages (Saccani and Natoli 2002; van Essen et al. 2010; Stender et al. 2012). No comprehensive analysis of the specific contribution of repressive histone marks towards PRG or SRG expression has been performed yet. Still, it is likely that basal repression mechanisms might play a more prominent role on the induction of late genes, as suggested by recent studies using a synthetic histone mimics to selectively block secondary gene expression in LPS-treated macrophages (Nicodeme et al. 2010).

As reported above, many secondary gene promoters require a nucleosome remodeling step before they can be occupied by TFs. This implies that, in order to promote their transcription, LPS stimulation must induce the recruitment of SWI/SNF complexes at these sites, possibly through the activity of one or more TFs able to bind compacted DNA. Studies in macrophages revealed that activation of IRF3 is a pre-requisite for nucleosome remodeling at a large group of late genes. LPS-induced expression of many SWI/SNF-dependent, CpG-poor genes was severely impaired in IRF3$^{-/-}$ macrophages, while SWI/SNF-independent genes were unaffected (Ramirez-Carrozzi et al. 2009). This was shown to reflect an essential role of IRF3 in promoting nucleosome remodeling and promoter accessibility at these genes in response to LPS stimulation. Altogether, these results indicate a high degree of functional specialization of the TFs activated by LPS in macrophages. Generic inflammatory TFs like NF-κB and AP-1 act to drive expression of genes with basally permissive promoters, typically encoding for broad mediators/regulators of environmental stress. This is consistent with the fact that these TFs are

broadly activated by multiple unrelated stimuli, such as serum, inflammatory cytokines, growth factors, or microbial products, which trigger globally overlapping gene expression programs; notably, the mean fold-induction of these genes after stimulation is relatively low (Bhatt et al. 2012), suggesting they might establish a "background" inflammatory state. On the other hand, highly specialized TFs like IRF3 become activated by very specific environmental stimuli, and selectively remove the nucleosome barrier at the promoter of a tightly regulated subset of inflammatory genes, such as those involved in antiviral responses. Consistent with their acute, and specialized, functions, these genes are generally upregulated at extremely high ratios (Bhatt et al. 2012). This functional specialization has fundamental implications as it allows achieving the stimulus-specific definition of transcriptional programs.

26.2.2 Cell-Type Specific Inducible Transcription

The capacity of different cell types to respond in a unique manner to identical environmental inputs represents the major feature of the functional specialization of cells in multicellular organisms. This observation is particularly relevant when considering cell types whose functional outputs are intimately linked to changes in environmental conditions, as is the case of immune system cells. Many efforts have been dedicated to understanding the determinants of cell-type specific gene expression, but only recently a global picture has begun to emerge that builds on the application of powerful genomic technologies to this biological problem. Notably, a substantial part of these findings were obtained in immune cell types; their implications are discussed below.

At least three conditions in which cell-specific gene expression is observed can be envisaged. First, *different cell types may respond differently to the same stimulus*. This is exemplified by the observation that macrophages and fibroblasts can both recognize and respond to LPS. However, specific induction of selected genes such as *Il12b* in macrophages (and dendritic cells) can be observed, in keeping with their specific role of antigen presenting cells (Smale 2012). Moreover, even when comparing the transcriptional responses to LPS of macrophages and dendritic cells, two distinct cell types so closely related that their definition as discrete cell entities has been questioned (Miller et al. 2012; Hume et al. 2013), cell-type specific gene expression has been described (Zanoni et al. 2009). Second, *the same cell type may respond differently to the same stimulus as the result of a different anatomical location*. This situation is intimately linked to macrophage biology, plastic cells that acquire specific phenotypic and functional features according to their tissue distribution. Accordingly, liver and brain macrophages (Kuppfer cells and microglia, respectively) show highly divergent responses to LPS stimulation, consistent with the existence of tissue-specific regulatory mechanisms (Crispe 2009; Ransohoff and Cardona 2010). Third, *the same cell type*, *in the same anatomical location*, *may respond differently to the same stimulus as a result of inter-individual or inter-species*

variations. Indeed, extensive intra-species differences in the transcriptional response to LPS have been recently reported in murine macrophages (Orozco et al. 2012) and human dendritic cells (Gat-Viks et al. 2013) derived from different individuals under identical conditions. Furthermore, massive inter-species discrepancies were described between the transcriptional profiles of murine and human macrophages during inflammatory conditions (Seok et al. 2013; Schroder et al. 2012).

Each of the situations described above can only partially be explained with a potential cell-specific expression of signaling molecules/regulators/TFs, either by developmental, environmental, or genetic causes. But even so, how do these differences translate into cell-specific gene expression programs?

26.2.2.1 One Genome, Hundreds of Phenotypes: The Role of Enhancers

Mammalian bodies are composed of about 200 specialized cell types. While sharing a common genome, each cellular unit displays a specific transcriptional output, clearly reflecting a differential usage of the coding and noncoding regulatory information. Due to their genomic distribution, promoters are largely invariant across cell types, i.e. to a large extent a given DNA region will act as a promoter for the same gene independently on the cellular context. Instead, enhancers are modular and their positioning is uncoupled from the TSS, implying that a given gene will be functionally associated with distinct enhancers in distinct cell types or temporal conditions. Accordingly, epigenetic marks of enhancers have been shown to strongly correlate with cell-type specific gene expression, suggesting they may be the primary drivers of specific transcriptional programs (Heintzman et al. 2009). A number of studies applied advanced genomic and computational approaches to annotate *cis*-regulatory elements in a variety of cell types, organisms, and environmental conditions (Visel et al. 2009; Kim et al. 2010; Akhtar-Zaidi et al. 2012; Bonn et al. 2012; Shen et al. 2012; Arnold et al. 2013; Ghisletti et al. 2010; Ostuni et al. 2013), leading to the identification of a massive, and still incomplete, catalogue of potential enhancers. Conservative estimates calculate that the human genome contains more than 1 million regions with features of enhancers (Consortium et al. 2012), globally regulating the expression of only 21,000 protein-coding genes (Lander 2011). The availability of such an enormous repertoire of enhancers implies that each cell type selectively uses only a fraction of all the possible regulatory information, thus achieving cell-specific regulation of gene expression: this selection is controlled by TFs.

26.2.2.2 The Myeloid TF Pu.1 and the Establishment of the Enhancer Repertoire

The essential feature of enhancers is their ability to act as platforms for the recruitment of multiple sequence-specific TFs through short motifs termed TF binding sites (TFBSs). In turn, TF binding at *cis*-regulatory elements drives transcriptional initiation or elongation of the target gene, likely through a direct

enhancer–promoter interaction enabled by the looping out of the intervening DNA (Deng et al. 2012). *Since not all TFs are active at the same time in a given cell*, *the cell-specific set of active enhancers is defined by the combinatorial DNA binding abilities of the active TFs and cofactors*. Accordingly, combinatorial occupancy by TFs was sufficient to predict the spatio-temporal activity of enhancers during *Drosophila* mesoderm development (Zinzen et al. 2009). There is increasing evidence that TFs differ considerably in their enhancer-shaping ability, as a reflection of their specific modes of interaction with chromatin. An extensive variability in the modes of TF binding at regulatory regions has indeed been described, including direct or indirect cooperativity mechanisms that result in specific transcriptional outputs (Spitz and Furlong 2012). However, a subset of lineage-determining TFs has a dominant role in the establishment of cell-type specific repertoires of enhancers, as exemplified by *Pu.1*, *the master regulator of macrophage differentiation* (Natoli 2010).

The hematopoietic-restricted ETS family member Pu.1 plays fundamental roles in multiple steps of blood cell development. Pu.1 is already active at early differentiation stages to control lymphoid and myeloid development, as shown by the complete absence of macrophages and B cells in Pu.1$^{-/-}$ mice (Scott et al. 1994). At the common myeloid progenitor (CMP) stage, Pu.1 promotes myeloid development by antagonizing with the megakaryocytic/erythroid TF GATA-1 (Galloway et al. 2005; Rhodes et al. 2005). Finally, lineage specification towards the monocytic lineage is achieved through the cooperation with TFs such as C/EBPα, C/EBPβ and IRF8, whereas granulocyte differentiation has been shown to require C-EBPε, GFI1 and, more recently, Jdp2 (Rosenbauer and Tenen 2007; Maruyama et al. 2012). Dynamic changes in Pu.1 expression levels during hematopoietic progression qualitatively affect specific developmental outputs; high expression levels of Pu.1 dictate macrophage differentiation, while tenfold lower concentrations of Pu.1 protein direct B cell development (DeKoter and Singh 2000). Therefore, both the combinatorial activity of partner TFs and its expression levels determine cell-specific Pu.1 functions.

ChIP-Seq analysis of genome-wide Pu.1 binding in macrophages revealed a pervasive genomic occupancy, mostly coinciding with distal DNA regions marked by the enhancer chromatin mark H3K4me1 (Ghisletti et al. 2010) (Fig. 26.1). Pu.1 binding was also consistently observed at transcriptionally competent promoters, suggesting it may globally regulate the three-dimensional organization of the chromatin in macrophages to allow enhancer–promoter interaction through homo- or heterotypic contacts (Natoli 2010). Notably, a direct ability of Pu.1 in driving a chromosome loop responsible for Pu.1 autoregulation has been recently reported in hematopoietic stem cells (HSCs) (Staber et al. 2013). The overwhelming majority of the H3K4me1-positive regions was occupied by Pu.1 in unstimulated macrophages, and ectopic expression of this TF in fibroblasts (which are Pu.1-deficient) was sufficient to induce the formation of macrophage-specific enhancers (Ghisletti et al. 2010). Furthermore, Pu.1 expression was able to restore H3K4me3 deposition at macrophage-specific promoters (Escoubet-Lozach et al. 2011). These findings clearly suggest a direct role for Pu.1 as a global organizer of the macrophage epig-

enome. It is worth noting that a very small fraction of macrophage regulatory elements were not bound by Pu.1 in the conditions analyzed. At least two scenarios can be envisaged to explain this observation. First, the absence of Pu.1 at these regions may reflect a multilineage priming of the enhancer repertoire preceding lineage commitment (Mercer et al. 2011). Second, Pu.1-negative enhancers might be autonomously specified by additional TFs involved in macrophage determination such as C/EBPβ (Heinz et al. 2010; Garber et al. 2012), C/EBPα (Jin et al. 2011), or IRF8 (Kurotaki et al. 2013). Interestingly, binding sites for these, as well as other TFs, were found to be significantly enriched at most macrophage enhancers, indicating that combinatorial binding of Pu.1 and other TFs represents a strategy to establish the macrophage-specific *cis*-regulatory repertoire (Ghisletti et al. 2010; Heinz et al. 2010; Pham et al. 2012; Ostuni et al. 2013). Genome-wide profiling of enhancers has also been reported in B cells. Consistent with a much lower concentration of Pu.1 in these cells, the genomic distribution of Pu.1 was largely different from that of macrophages, leading to the establishment of distinct enhancer repertoires (Heinz et al. 2010). Furthermore, formation of B cell-specific enhancers by Pu.1 required an extensive functional cooperation with multiple TFs specifically expressed in B cells such as E2A and PAX5 (Heinz et al. 2010; McManus et al. 2011).

These and subsequent studies provided the basis for a general model describing how cell-type specific enhancer repertoires are established and maintained (Fig. 26.2). During early stages of development, restricted sets of lineage-determining TFs (exemplified by Pu.1 and C/EBPβ in macrophages) functionally cooperate to occupy a large number of sites along the genome. These early binding events occur at "virgin" regions of DNA that are characterized by an inactive chromatin conformation and high nucleosome occupancy, features that impede the binding of the vast majority of TFs operating only within a permissive chromatin environment. Instead, the above-mentioned TFs retain the ability to bind to their target DNA sequences in a native context, and are therefore referred to as *pioneer factors* (Zaret and Carroll 2011). Examples of pioneer TFs include Pu.1 and C/EBPβ in macrophages, dendritic cells, and B cells (Ghisletti et al. 2010; Heinz et al. 2010; Garber et al. 2012), FoxA1 and GATA-4 in liver development (Cirillo et al. 2002; Lupien et al. 2008), and Oct4, Sox2, and Klf4 in human induced pluripotent stem (iPS) cells (Soufi et al. 2012). A common feature of pioneer TFs is to facilitate successive binding of non-pioneer TFs through a direct/indirect recruitment of chromatin remodelers and histone modifying enzymes. Consistent with this definition, Pu.1 was shown to induce nucleosome depletion and H3K4me1 deposition at regulatory elements when ectopically expressed in Pu.1-deficient cells (Ghisletti et al. 2010; Heinz et al. 2010), possibly through the recruitment of Brg1-containing BAF complexes (Hu et al. 2011), Mll3/Mll4 histone methyltransferases (Herz et al. 2012), and p300/CBP HATs (Ghisletti et al. 2010) (Fig. 26.2).

In conclusion, in mammals cell-type specific enhancer repertoires are largely established during development through the instructive activity of the same lineage-determining TFs that define and maintain cell identity. This provides a mechanistic link between lineage determination and transcriptional control by TFs (Natoli 2010).

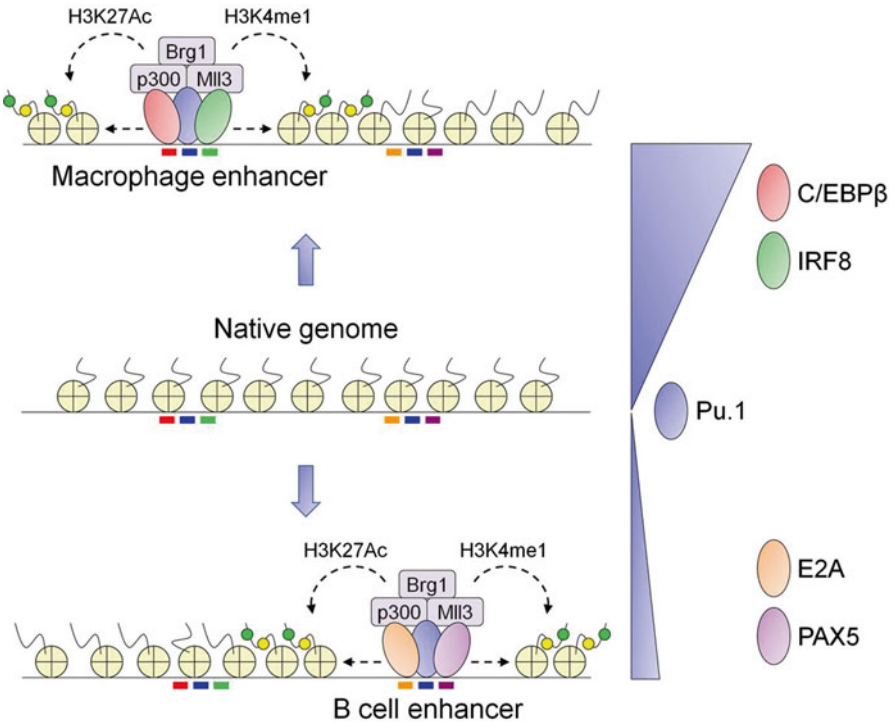

Fig. 26.2 Establishment of cell-specific enhancer repertoires by Pu.1. During macrophage development, the lineage-determining TF Pu.1 is expressed at very high levels, together with the macrophage-specific pioneer TFs C/EBPβ and IRF8. These TFs operate combinatorially to access their binding sites (*colored boxes*) in a native chromatin context. Recruitment of nucleosome remodeling complexes and histone modifying enzymes leads to enhancer formation. In B cells, where the expression levels are much lower, Pu.1 cooperates with additional TFs such as PAX5 and E2A to establish the B cell-specific enhancer repertoire. *Green* and *yellow circles* represent H3K27Ac and H3K4me1, respectively (color figure online)

26.2.3 Integration of Cell- and Stimulus-Specific Inputs at Enhancers

26.2.3.1 Dynamic Alterations of the Enhancer Repertoire Underlie Macrophage Functions

At a given time, or in a given condition, specific combinations of histone modifications define at least three activity states of enhancers: (1) *active* (high H3K27Ac, no H3K27me3), (2) *poised* (no H3K27Ac, no H3K27me3), and (3) *repressed* (no H3K27Ac, high H3K27me3) (Creyghton et al. 2010; Rada-Iglesias et al. 2011). Additional features of active enhancers include occupancy by Pol II, leading to the

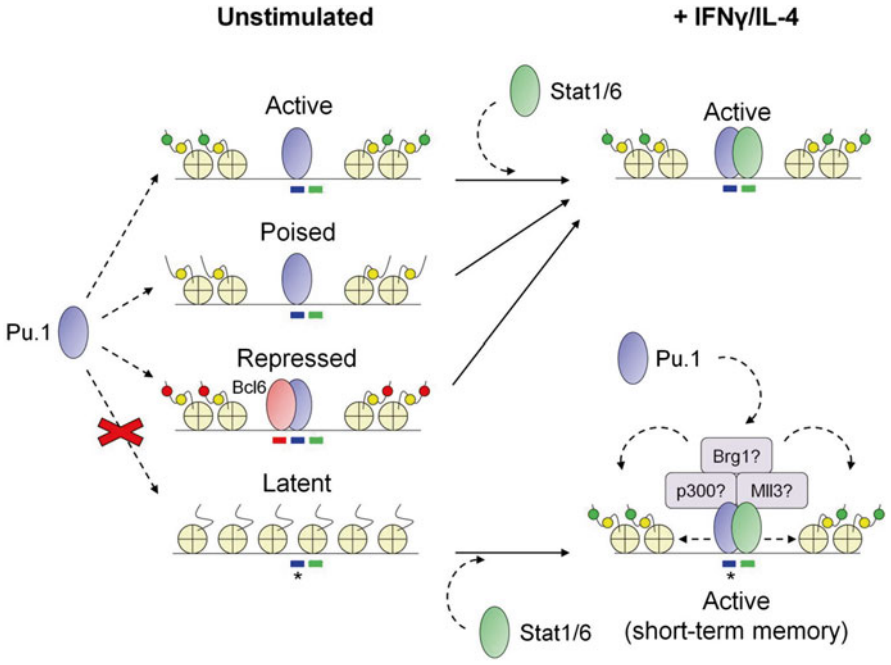

Fig. 26.3 Stimulus-dependent alterations of the enhancer repertoire. In macrophages, Pu.1 constitutively marks active, poised, and repressed enhancers. Upon IFNγ (or IL-4) administration, the TFs Stat1 (or Stat6) bind to these pre-established regions to qualitatively alter their functional state (e.g., to activate them). Conversely, latent enhancers harboring a low affinity Pu.1 binding site (represented by *an asterisk*) are not bound by Pu.1 in the basal state and do not display known enhancer-associated features. In response to IFNγ/IL-4 stimulation, activated Stat1/6 exert a pioneer-like activity to promote Pu.1 recruitment, nucleosome remodeling, and deposition of active enhancer marks at these regions. Latent enhancers are remarkably stable, and may mediate a short-term transcriptional memory (see text). *Green*, *yellow and red circles* represent H3K27Ac, H3K4me1, and H3K27me3, respectively (color figure online)

generation of short, bidirectional RNAs of unknown function, termed enhancer RNAs (eRNA) (De Santa et al. 2010; Kim et al. 2010; Natoli and Andrau 2012). Conversely, repressed enhancers are often bound by transcriptional corepressors, exemplified by Bcl6 in macrophages (Barish et al. 2010) (Fig. 26.3).

Consistent with the massive transcriptional rearrangements observed after LPS stimulation in macrophages or dendritic cells, thousands of enhancers acquired or lost p300 binding, H3K27Ac and H4Ac, thus transitioning between different activity profiles (Ghisletti et al. 2010; Ostuni et al. 2013; Garber et al. 2012; Chen et al. 2012). Activation of LPS-responsive enhancers coincided with the acute recruitment of the pro-inflammatory TFs NF-κB, AP-1, and IRFs to regions that in most cases were already marked by Pu.1 (Ghisletti et al. 2010; Garber et al. 2012; Ostuni et al. 2013). Similar findings were obtained after stimulation of macrophages with pro- or anti-inflammatory molecules including the M1/M2 inducers IFNγ and

IL-4 (Fig. 26.3). These cytokines triggered the inducible recruitment of the TFs STAT1 and STAT6 mostly at, but not limited to, pre-marked enhancers (Ostuni et al. 2013). Importantly, IFNγ or IL-4-induced deposition of H3K27Ac at enhancers was impaired in STAT1$^{-/-}$ or STAT6$^{-/-}$ macrophages, indicating that inducible TFs have a causal role in mediating enhancer activation through the recruitment of histone modifiers (Ostuni et al. 2013). Inducible recruitment of stimulus-activated TFs at pre-established enhancers marked by lineage-determining TFs was observed in multiple unrelated experimental systems. Treatment of HeLa cells with IFNγ triggered STAT1 recruitment at regions marked by H3K4me1 (Heintzman et al. 2009). FoxA1 was found to organize the enhancer repertoire in mammary epithelial and prostate cancer cells, respectively restricting estrogen receptor α (ERα) and androgen receptor (AR) recruitment at predefined genomic sites (Hurtado et al. 2011; Wang et al. 2011). Finally, transforming growth factor β (TGFβ) stimulation of different cell types resulted in the recruitment of Smad2/3 to distinct sets of regulatory elements determined by cell-type specific TFs (Mullen et al. 2011).

These findings clearly indicate that, by establishing a cell-type specific repertoire of accessible regulatory elements, lineage-determining TFs set the stage for the subsequent binding of stimulus-induced TFs (Fig. 26.3). Not only this model provides a simple explanation for how cell- and stimulus-specific inputs are integrated at regulatory elements (Natoli 2010), but also allows TFs involved in inducible responses to be classified into different hierarchical layers, based on their modes of action at *cis*-regulatory regions (Garber et al. 2012): *pioneer TFs* (e.g., Pu.1, C/EBPβ) initiate chromatin remodeling by opening previously inaccessible sites; *primer TFs* (e.g. JunB) occupy these elements in a relatively static manner and prime future inducible responses, possibly by reinforcing the accessible chromatin state (Biddie et al. 2011); *effector TFs* (e.g. NF-κB) activated by stimulation bind to specific subsets of already accessible regulatory elements to direct the expression of specific subsets of genes.

26.2.3.2 Expansion of the Enhancer Repertoire by Stimuli: Latent Enhancers

Restrictive interpretations of the data described above led to the emergence of a prevailing concept: that the enhancer repertoire is completely established during development by lineage-determining TFs, and that all transcriptional regulatory events in differentiated cells occur within this predetermined landscape. As a corollary, this model implies that the effect of external stimulations in terminally differentiated cells is limited to a qualitative modulation of the activity state of pre-existing enhancers, and that no new enhancers can be formed.

Recent studies provide direct experimental evidence against this view. A systematic analysis of the dynamic alterations of the enhancer repertoire in differentiated macrophages revealed that, in addition to expected changes in the activity state of pre-existing enhancers, environmental stimulation also triggered the de novo deposition of enhancer marks at previously silent sites (Ostuni et al. 2013). These elements

were termed *latent enhancers* and were defined as genomic regions that are unmarked and unbound by TFs in terminally differentiated cells, but acquire H3K4me1 and TF occupancy after stimulation (Fig. 26.3). Stimulus-dependent formation of latent enhancers in macrophages was observed in response to a panel of unrelated stimuli including TLR agonists, pro- and anti-inflammatory cytokines (e.g., IFNγ and IL-4), indicating that expansion of the available *cis*-regulatory repertoire represents a general hallmark of cellular responses to environmental changes. Different stimuli induced the unveiling of distinct sets of latent enhancers, in turn correlating with stimulus-specific gene expression programs (Ostuni et al. 2013). Mechanistically, latent enhancers were formed through the combined action of stimulus-activated and lineage-determining TFs, but in a rather peculiar manner. Upon IFNγ or IL-4 stimulation of macrophages, Pu.1 binding at latent enhancers required an initial step of STAT TFs occupancy, reflecting the presence of low affinity Pu.1 binding sites at these regions. Therefore, Pu.1 does not act as a pioneer TF at IFNγ- or IL-4-induced latent enhancers but rather reinforces the enhancer-shaping activities of STAT1 or STAT6 (Fig. 26.3). Importantly, STAT TFs have been recently reported to direct the generation of the active enhancer repertoires as well as the global genomic organization of $CD4^+$ T helper cells (Vahedi et al. 2012; Hakim et al. 2013), strongly indicating they might not simply act as signaling TF as previously considered. Upon removal of the inducing stimulus, latent enhancer did not return to a silent state; instead, a large fraction of them retained the H3K4me1 mark for days after cytokine washout. Persistent H3K4me1 maintained latent enhancers in a poised state, allowing faster and stronger deposition of H3K27Ac upon secondary stimulation, consistent with the reported ability of H3K4me1 to prime enhancers for recruitment of HATs (Jeong et al. 2011). Importantly, increased re-acetylation of latent enhancers correlated with a significantly augmented target gene expression upon secondary stimulation, indicating that these genomic elements might confer a short-term transcriptional memory to exposed cells. These findings are consistent with the observation that repeated LPS administration to macrophages resulted in the hyperinduction of a subset of non-tolerant genes (Foster et al. 2007). Furthermore, they may provide a mechanistic framework underlying the emerging concept of "innate immune memory," or "trained immunity" (Netea et al. 2011), according to which initial infectious exposure confers protective immunity against reinfection via reprogramming of monocytes (Quintin et al. 2012). In general, cooperation between signaling-activated and lineage-specific TFs to establish new enhancers and modulate downstream gene expression provides a mechanism for cell plasticity and the reprogramming of macrophage responses to environmental stimuli that plays a key role in innate immunity, inflammation, and autoimmune diseases (Ivashkiv 2012).

Therefore, environmental changes shape the enhancer repertoire both quantitatively, via activation or repression of pre-existing enhancers, and qualitatively, via de novo formation of latent enhancers (Fig. 26.3). These findings suggest that stimulus-specific expansion of the *cis*-regulatory repertoire provides an epigenomic memory of the exposure to environmental agents.

26.3 Concluding Remarks

The advent of next generation sequencing technologies allowed us to glimpse into the complexity of how the regulatory information encoded in the genome is functionally deployed to specify transcriptional outputs. In this context, macrophage proved instrumental to uncover some of these fundamental principles. Given their plastic properties and their involvement in physiological and pathological processes, these cells will definitely continue to be at the center of the stage. A number of crucial issues related to the control of transcription in macrophages remain to be addressed. Specifically, what is the functional *cis*-regulatory repertoire of macrophages in vivo? How does it change between different macrophage populations and between macrophages located in different tissues? How is it altered in pathological conditions? Furthermore, what are the target genes of enhancers? How do mutations in enhancers (including common single nucleotide polymorphisms) alter their functionality and thus impact on macrophage function and inflammation? How does this contribute to human disease? The kind of investigations described in this chapter represents the ground to tackle and answer at least a fraction of these questions.

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Chapter 27
Role of MicroRNA in Macrophage Activation and Polarization

Graziella Curtale and Massimo Locati

27.1 MiRNA: From the Biogenesis to the Function

It has been estimated that only 2 % of the mammalian genome encodes for protein products, whereas 70–90 % is extensively transcribed to produce a large transcriptome of non-coding RNA species (ncRNA). The unexpected pervasive transcription recently revealed by improved RNA-seq technologies has challenged the traditional view of RNA as merely intermediary between genes and proteins and has identified ncRNA as functional RNA species involved in the regulation of fundamental biological processes. Since their first discovery in 1993 (Wightman et al. 1993; Lee et al. 1993), the list of functional ncRNA classes is growing and to date it appears evident that microRNA (miRNA), which represent the most comprehensive and well characterized class of small ncRNA, are just the tip of the iceberg. MiRNA are a class of small evolutionary conserved ncRNA which regulate translation and stability of several target mRNA in a sequence-specific manner. They can be encoded as single miRNA or be organized in cluster of multiple miRNA. They have been identified in intergenic regions of the genome (Olena and Patton 2010), where they are transcribed as standing alone units, and in other cases at the level of annotated transcripts, located in introns of protein-coding genes; in the latter case their expression is often co-regulated with that of the host gene. They originate from a primary transcript of ~1 kb length called primary miRNA (pri-miRNA), processed in the nucleus by the RNAse III enzyme Drosha and DGCR8 into an hairpin precursor called precursor miRNA (pre-miRNA) of about 60 nt in length. The pre-miRNA is then exported to the cytoplasm where it is cleaved by the RNAse III Dicer to

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generate imperfect miRNA–miRNA* duplexes (Dueck et al. 2012). The preferential selection of one miRNA strand depends, at least in part, the thermodynamic instability of the mature miRNA strand (Hu et al. 2009) by leading to the generation of a mature miRNA of ~22 nt in length, incorporated into the RNA-induced silencing complex (RISC). Within the RISC, the mature miRNA interacts with members of the argonaute (Ago) proteins and drives Ago proteins and other associated factors to partially complementary target sites, mainly located in the 3′UTR of the mRNA targets (Chekulaeva and Filipowicz 2009; Filipowicz et al. 2008), leading to their post-transcriptional repression (Chekulaeva and Filipowicz 2009; Filipowicz et al. 2008). The precise mechanism of such down-regulation is not fully understood, but is largely dependent on the extent of base-pairing complementarity between the miRNA and the respective mRNA target (Lee and Shin 2012). Since the first evidence of miRNA deregulated expression in cancer cells (Calin et al. 2002), it has become apparent that miRNA are indeed key regulators of cellular functions in different aspect of cell biology, from cell development to apoptosis, cell cycle, and differentiation (Lodish et al. 2008; Bartel and Chen 2004). From the perspective of innate immunity, miRNA have been extensively studied both in physiological and pathological contexts. They are differentially expressed in immune cells and are able to target proteins involved in the modulation of panel of genes involved in myeloid cell maturation, inflammatory pathways, and macrophage polarization processes. At the molecular level mRNA targets show in most cases a relatively mild down-regulation at the protein level (O'Connell et al. 2012) (from 1.2 to 4-fold) as miRNA do not act as on–off molecular switches but rather fine tune the expression of multiple target genes involved in a common biological processes. Specific sets of miRNA have also been demonstrated to work in concert with other regulatory molecules [i.e., transcription factors and other emerging classes of ncRNAs (Pagani et al. 2013)] to affect the expression of key components of signaling pathways. The proper combination of these mechanisms results in a precise and timely modulation of cellular responses, affecting strength, location, and timing of the immune response (Bhaumik et al. 2008; Baek et al. 2008).

27.2 MiRNA and Myeloid Cells Differentiation

More than 900 microRNAs (miRNA) have been identified in mammalian cells and more than 100 of them are expressed in the hematopoietic tissue, where different subsets of miRNA have been directly implicated in controlling the development and function of the innate (myeloid) and adaptive (lymphoid) immune system (Chen et al. 2004). The first direct evidence demonstrating a role of miRNA in hematopoiesis has been provided by conditional $Dicer^{lox/lox}$ gene-targeted murine models in which Dicer was selectively depleted from the hematopoietic system. Although *Dicer*$^{-/-}$ hematopoietic stem cells (HSCs) persisted in recipient mice for months, there was a significant reduction in the primitive LKS ($Lineage^{-}/cKit^{+}/Sca\text{-}1^{+}$) progenitor population as well as all mature lineages, particularly myeloid cells

(Guo et al. 2010). The development of myeloid cells is a multi-step process, which begins in the bone marrow, where HSCs are located in microenvironmental niches (Akashi et al. 2000). Several endogenous and exogenous cues guide the decision fate of these cells, regulating their clonogenic activity and stemness (Starnes and Sorrentino 2011; Adolfsson et al. 2001). Recently, miRNA have been described as key regulators of hematopoiesis (El Gazzar and McCall 2012; Gangaraju and Lin 2009) along with transcription factors. Specific signatures of differentially expressed miRNA have been identified as associated with distinct phases of lineage development and differentiation (El Gazzar and McCall 2012; O'Connell et al. 2011). Comparative studies of miRNA expression profiling in $CD34^+$ hematopoietic progenitor cells, comprising short-term HSCs and multipotent myeloid progenitors (MMPs), have identified specific miRNA expressed in these cells, having critical roles in the early hematopoiesis and lineage-restriction process. In particular, miR-125a (member of miR-125a/99b/let7e cluster) and miR-29a promote HSC maintenance: miR-125a regulates HSC survival by inhibiting the pro-apoptotic factors BAK1 (BCL2-homologous antagonist/killer), KLF13 (kruppel-like factor), and BMF (BCL2-modifying factor) (Guo et al. 2010); miR-29a over-expression in HSCs and MPPs promotes self-renewal and blocks progression towards more committed myeloid lineage populations (Han et al. 2010). Other specific set of miRNA, represented by miR-221/miR-222 cluster, miR-10 and miR-196b, promotes generation of lineage-restricted progenitors: the miR-221/miR-222 cluster limits self-renewal capacity of HSCs and MPPs by inhibiting c-Kit expression (Felli et al. 2005; Forrest et al. 2010), while miR-10 and miR-196b repress Hox genes in short-term HSCs and are in turn modulated by MLL (transcription co-factor mixed lineage leukemia), a master regulator during development and hematopoiesis (Popovic et al. 2009). MiR-29a is highly expressed in HSCs and down-regulated in MPP and differentiated myeloid cells (Han et al. 2010) and miR-126 is another example of miRNA critically involved in promoting the generation of lineage-restricted progenitor CMP (common myeloid progenitor) from MPP (Shen et al. 2008). The decision of CMPs to generate granulocytic–monocytic lineage (GMPs) or megakaryocyte–erythroid progenitors follows changes in transcription factors (TFs) expression profile. Transcription factors are the primary determinants that resolve mixed lineage patterns of gene expression by activating lineage appropriate genes and repressing alternate genes; thus the low level expression of specific TFs in CMPs reflects priming stages at which lineage commitment process remains flexible and changes in TFs expression patterns along with miRNA counterparts most likely dictate all decisions of myeloid lineage development and differentiation (Laslo et al. 2008). The generation of GMPs is mainly driven by two master regulators: PU.1 and C/EBPα (Dahl et al. 2003). PU.1, a member of ETS family of transcription factors, is critical for the specification of the CMP from HSCs, by inhibiting GATA-1 expression, which is required for megakaryocyte–erythroid progenitors (MEPs) differentiation (Nutt et al. 2005). C/EBPα synergizes with PU.1 to maintain granulocyte–macrophage lineage commitment (Scott et al. 1994). MiRNA differential expression during various stages of myeloid development is strictly dependent

on specific TFs involved in myelopoiesis (Starnes and Sorrentino 2011). On the other hand, miRNA act in concert with lineage-specific transcription factors to regulate myeloid-derived innate immune cells, including monocytes and granulocytes (Fazi et al. 2005; Fontana et al. 2007). For example, PU.1 and C/EBPβ induce the expression of miR-223, whose levels progressively rise during successive stages of granulocytic differentiation, whereas NFI-A, that is directly targeted by miR-223, itself represses this miRNA, thereby forming a positive-autoregulatory circuit (Fazi et al. 2005; Johnnidis et al. 2008). MiRNA and TFs function in antagonistic mode to repress each other's targets. MiR-155, which is induced by NF-κB and is highly expressed in differentiated immune cells including monocytes and macrophages, regulates PU.1 and C/EBPβ levels, thereby modulating the transcriptional profile of the cell (O'Connell et al. 2008; Georgantas et al. 2007). The growth factor M-CSF (macrophage-colony stimulating factor) and its receptor (M-CSFR) have an essential role in development, differentiation, and maturation of monocytes/macrophages. M-CSF promotes differentiation of $CD34^+$ hematopoietic progenitors into monocytes by inducing changes in the expression of specific miRNA and TFs, which regulate each other's expression through a feed-forward loop and feedback loop (Georgantas et al. 2007). In particular, three main miRNA: miR-106a (member of the miR-106a-92 cluster), miR-17-5p, and miR-20a (members of the miR-17-92 cluster) are expressed at high levels in early myeloid progenitors and are down-regulated during monocytes differentiation (Fontana et al. 2007). Notably, these miRNA bind to the 3′untraslated region of AML-1 (acute myeloid leukemia-1), a transcription factor involved in monocytic lineage differentiation, and repress its translation. During the differentiation process, expression of miR-106a, miR-17-5p, and miR-20a decreases and their reduction levels activate AML-1 expression, thus promoting monocytic progenitor differentiation (Fontana et al. 2007). Indeed, AML-1 has been shown to induce monocyte differentiation by inducing the expression of M-CSFR and also binds to miR-106a, miR-17-5p, and miR-20a promoter regions and represses their transcription. Thus, the decrease of miR-106a, miR-17-5p, and miR-20a expression establishes a negative-feedback loop mechanism, involving increase of AML-1 and M-CSFR expression and the subsequent reduction in the expression of these miRNA. A second example of integrative cooperation between TFs and miRNAs in monocytic differentiation process is provided by miR-424, whose expression is induced in GMP by the myeloid transcription factor PU.1. MiR-424 promotes mononuclear phagocyte differentiation by inhibiting NFI-A (Rosa et al. 2007), whose high expression levels in GMPs maintain their undifferentiated state, whereas its down-regulation in the context of AML-1 feedback loop (via miR-17-92 and miR-106a) or PU.1 feed-forward loop (via miR-424) promotes monocytic progenitor differentiation. Thus, the interplay between these molecular switches dictates the commitment of myeloid progenitor towards monocytic lineage. MiRNA and TFs establish complex regulatory networks, by forming lineage-specific regulatory loops, where both the miRNA and the TFs regulate each other's expression (Popovic et al. 2009).

27.3 MiRNA and Macrophage-Mediated Inflammatory Response

After maturation in the bone marrow, myeloid cells are subject to activation in response to pathogens, resulting in the induction of an inflammatory response. The activation of myeloid cells is induced by inflammatory stimuli and/or recognition of pathogen-associated molecular patterns by pattern recognition receptors including Toll-like receptors (TLRs), and leads to extensive intracellular signaling and changes in transcriptional gene expression program. Several miRNA are induced in monocytes and macrophages upon TLR engagement, with subtle differences in miRNA expression profiles observed depending on the TLR agonist used (O'Neill et al. 2011). As for other TLR-dependent gene, miRNA can be classified as early or late response miRNA: early miRNA, including miR-155, miR-146a, and miR-9, are rapidly upregulated in macrophages, whereas late response includes miR-21 (O'Neill et al. 2011), miR-187 (Rossato et al. 2012), and miR-146b. The induction of TLR-responsive miRNA is also under strict control. Conversely, how TLR signaling decreases miRNA expression is largely unknown, though it is generally assumed that transcriptional repression and destabilization of miRNA transcripts are involved. For example, miR-155, one the first miRNA described as an important mediator in the macrophage activation process, is highly induced in response to TLR agonists or type I interferons, in both monocytes and macrophages of human or mouse origin (O'Connell et al. 2007) and is in turn negatively regulated by IL-10, a key anti-inflammatory cytokine. The IL-10-dependent inhibition of miR-155 increases the expression of SHIP1 (McCoy et al. 2010) (Src homology 2 domain-containing inositol 5′-phosphatase 1), a negative regulator of TLR4 signaling directly targeted by miR-155. Furthermore, miR-21, also expressed in inflammatory macrophages, controls inflammation by downregulating the translation of the pro-inflammatory PDCD4 (Sheedy et al. 2010) (programmed cell death 4), a tumor suppressor gene which inhibits IL-10 expression. The resulting increase of IL-10 levels promotes an anti-inflammatory response by a feedback mechanism which contributes to the fine-tuning of TLR4 signaling. Indeed, the initial increase of miR-155 levels in response to TLR4 activation downregulates the expression of SHIP1 and strengthen the TLR4 signaling. Later in the response, the IL-10 induction by miR-21 decreases PDCD4, an inhibitor of IL-10 translation, forming a feedback loop mechanism that affects miR-155 levels, thereby restoring SHIP1 levels and limiting TLR4 signaling. Other miRNA highly expressed in both human and murine macrophages upon LPS challenging in a NF-κB-dependent manner and involved in negative-feedback loops are represented by miR-146a (Taganov et al. 2006) and miR-9 (Bazzoni et al. 2009). In particular, miR-9 directly downregulates the NF-κB subunit p50, whereas miR-146a indirectly affects NF-κB activity by downregulating the expression of IRAK-1 and TRAF6, two key adaptor proteins of the TLR signaling cascade required for NF-κB activation. Recently, a new set of anti-inflammatory miRNA, expressed in monocytes and macrophages has been described as part of the regulatory mechanism which dictates the resolution of inflammation. In

particular, miR-187 (Rossato et al. 2012) and miR-146b (manuscript in press) have been described as IL-10-dependent miRNA, expressed as consequence of the endogenous production of IL-10 at late times after LPS challenge. These miRNA might contribute to the resolution of inflammation through the direct inhibition of NFKBIZ and TNFα (miR-187) and the multiple targeting of TLR4, IRAK1, MyD88, and TRAF6 (miR-146b). Furthermore, the over-expression of miR-146b results in the global down-regulation of pro-inflammatory cytokines, including TNFα, IL-6, CXCL8, and IL-12p40. These few examples provide a paradigm for the action of miRNA in the context of macrophage-driven inflammation. Overall, it is becoming apparent that rather than shutting down the TLR signaling pathway, miRNA modulate TLR activity through the multiple targeting of different components of their signaling cascade. This emerging regulatory level relies on the simultaneous action of several miRNA, whose sequential expression is important to control the strength and timing of the inflammatory response.

27.4 MicroRNA and Macrophage Polarization

Macrophages residing in distinct tissue microenvironments display divergent phenotypes and functions. Such heterogeneity is defined by the identity of the precursor from which the macrophages derive and by the action of microenvironment factors to which they are exposed. When engaged, different macrophage receptors activate distinct intracellular molecular pathways and, consequently, activation states in macrophages. In schematic terms, macrophages can undergo either classical (M1) or alternative (M2) activation (Martinez et al. 2008). Bacterial products (including LPS) and several pro-inflammatory cytokines (including IFNs and TNFα) promote M1 activation and induce production of high levels of pro-inflammatory (TNFα, IL-6, IL-1, IL-12) and angiostatic cytokines (CXCL10, IL-12). By contrast, cytokines (IL-4/IL-13, IL-10), glucocorticoids, and TGFβ favor the induction of an alternative (M2) activation state that is associated with lower secretion of pro-inflammatory cytokines and enhanced expression of tissue-remodeling and pro-angiogenic factors. Classically activated macrophages respond to intracellular bacteria/viruses, whereas alternatively activated macrophages sense parasites and promote angiogenesis and wound healing (Mantovani et al. 2013). Classic and alternative activation represent extremes of a phenotypic continuum; indeed, intermediate/mixed activation states largely predominate in vivo.

Expression profiling studies have reported the differential expression of miRNA between M1 and M2 macrophages, thus supporting the general idea that miRNA are involved not only in macrophage-mediated inflammatory response, but also in the modulation of macrophage polarization. Several miRNA are induced upon either type of macrophage activation, with a general increase of their expression during the monocyte differentiation to macrophage. The effective role of miRNA in this process has been largely unexplored; to date only few studies have characterized the functional role of individual miRNA in this context. Some miRNA, like miR-155,

preferentially expressed in classical activated macrophages, primarily sustain classical activation by enhancing pro-inflammatory signaling, or by attenuating alternative activation. The fundamental role of miR-155 in the macrophage polarization process has been demonstrated by gain of function studies, showing that miR-155 enforced expression in alternatively activated macrophages is sufficient to reprogram these cells toward a more pro-inflammatory phenotype (He et al. 2009). It promotes classical macrophage activation by down-modulating SOCS1 and BCL6; moreover, it directly targets IL-13RA1, the IL-13 receptor, involved in the induction of M2 polarization (Martinez-Nunez et al. 2011). Conversely, other miRNA, like miR-511-3p and miR-378-3p are mainly expressed in M2 polarized macrophages and attenuate alternative activation (Ruckerl et al. 2012; Squadrito et al. 2012). miR-511-3p is located in the fifth intron of both human and mouse MRC1 gene, that encode for the mannose receptor (also known as CD206), a marker of M2 polarized macrophages, and it downregulates ROCK2 (Rho-associated coiled-coil containing protein kinase 2), a serine threonine kinase that phosphorylates IRF4, a transcription factor that promotes M2 polarization (Squadrito et al. 2012). MiR-378-3p, instead is induced upon an IL-4 stimulation and is involved in a negative-feedback loop that results in the attenuation of the alternative activation of macrophage, by targeting the PI3K/Akt1 pathway (Ruckerl et al. 2012). A third miRNA, recently described in the context of M2-polarized macrophages, is let-7c (Banerjee et al. 2013), whose expression is significantly reduced by LPS stimulation and increased when M1 macrophages are converted to M2 macrophages. It has been demonstrated that let-7c suppresses polarization of macrophages to the M1 phenotype and enhances M2 polarization, even though the molecular targets directly affected by this miRNA are yet unraveled. Thus, it is likely that miRNA are involved in shaping the M1/M2 balance and provide a general mechanism for limiting macrophage activation in both alternative and classical activation settings.

27.5 MiRNA and Tumor-Associated Macrophages

Deregulation of miRNA can drive oncogenesis, tumor progression, and metastasis by acting cell-autonomously in cancer cells. However, solid tumors are also infiltrated by large amounts of non-neoplastic stromal cells, including macrophages, which express several active miRNA. Tumor-associated macrophages (TAMs) promote malignant progression by enhancing angiogenesis, tumor cell migration, suppressing antitumor immunity, reducing host survival, and facilitating tumor cell invasiveness and metastatic programming of neoplastic tissue. Multiple cytokines expressed in the tumor microenvironment, such as IL-4, IL-10, transforming growth factor (TGFβ), TNFα, CCL2, 17-CSF, vascular endothelial growth factor (VEGF), and angiopoietin 2 (ANG2), are known to regulate macrophage development, recruitment, differentiation, and/or M1/M2-like activation in cancer (Ruffell et al. 2012; Sica and Bronte 2007). Many of these cytokines modulate miRNA expression and activity in different cell types, including cultured monocytes/macrophages, but

little is known about their ability to control miRNA function in TAMs (Squadrito et al. 2013). Furthermore, multiple miRNA (e.g., miR-23, miR-24, miR-26, miR-103, and miR-181) are induced by hypoxia, which is a hallmark of the tumor microenvironment (Kulshreshtha et al. 2007). However, the expression of such hypoxia-inducible miRNA in TAMs, and their relevance for the effector functions of TAMs, remain to be investigated. It is increasingly evident that miRNA modulate macrophage response to cancer by affecting macrophage polarization in the tumor microenvironment. To the best of our knowledge, miR-511-3p is the first miRNA whose activity and function was studied directly in TAMs in vivo (Squadrito et al. 2012). As previously mentioned, miR-511-3p is an intronic miRNA, coexpressed with its host gene MRC1 in M2 polarized macrophages. Alternatively activated TAMs express the macrophage mannose receptor, MRC1, and upregulate several genes that confer them protumoral functions. The induction of MRC1 expression triggers a negative-feedback response orchestrated by miR-511-3p, which tunes down the protumoral gene signature of $MRC1^+$ TAMs and inhibited tumor growth. Indeed, miR-511-3p downregulates TAM expression of multiple genes with protumoral effector function, involved in extracellular matrix (ECM) synthesis and remodeling, including collagens and other fibrous proteins, proteases, and scavenger receptors (Squadrito et al. 2012). Because $MRC1^+$ TAMs represent a major component of the perivascular tumor stroma and support vascular morphogenesis in tumors, modulation of ECM-protein synthesis/remodeling by miR-511-3p in $MRC1^+$ TAMs may have the potential to influence ECM dynamics in the perivascular microenvironment. Consistently, miR-511-3p over-expression in TAMs altered the morphology of intratumoral blood vessels, possibly as a consequence of changes in the biophysical properties of the perivascular ECM (and/or in the levels of TAM-derived angiogenic factors). These findings suggest that miR-511-3p functions as an endogenous relay that attenuates the protumoral functions of alternatively activated TAMs. Recent evidence also point out to miR-155 as a key miRNA involved in the TAM biology; as previously discussed it is induced by inflammatory stimuli such as TLR ligands and inflammatory cytokines. MiR-155 is also upregulated in $CD11c^+$ pro-inflammatory TAMs, whereas is not expressed in the $MRC1^+$ protumoral macrophages, suggesting that it is activated in a subset of TAMs with pro-inflammatory and antigen presenting functions. This created an imbalance towards a protumoral microenvironment as evidenced by a lower proportion of $CD11c^+$ TAMs, reduced expression of activation markers, and skewing of immune cells within the tumor towards an M2/Th2 response. This is consistent with the impaired capacity of miR-155-deficient macrophages to establish a pro-inflammatory microenvironment within the tumor, likely resulting in a reduction of antitumor immune response. Altogether the findings outlined above suggest that endogenous miRNA tightly regulate the macrophage response to microenvironmental cues and may operate to establish the pro-versus antitumoral functions of TAMs. It is increasingly apparent that macrophage differentiation and activation is likely to be modulated by the concerted action of multiple miRNA, which significantly affect key signaling pathways that are co-opted in cancer.

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Part VI
Systems Biology of Macrophages

Chapter 28
The Macrophage Transcriptome

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28.1 Abstract

Macrophages are specialized but versatile cells that participate in a range of physiological and immune related processes. The macrophage repertoire of coding and regulatory RNA provides tools to understand cell identity, cell function, role in disease, and ultimately define cell specific therapeutic targets. Modern tools make it possible to quantify and compare global RNA levels. With this vast information a neologism of the decade, the suffix "*ome*" has been combined with "*transcript*" to form "transcriptome," a new word to define the totality of transcripts that characterize a cell. In this chapter we discuss the macrophage transcriptome and how its definition is contributing to a deeper understanding of this cell identity and function.

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28.2 Background

Macrophages are heterogeneous cells that play a key role in tissue homeostasis and defense against invading pathogens. To achieve these tasks they occupy virtually every tissue in the body, forming a complex network of resident cells that sense physiological, infectious, and pathological signals. When needed, additional macrophages deriving from the pool of circulating monocytes add to the crew of resident macrophages to counteract the changes in the tissue and restore normality, which not only involves eliminating danger signals but also clearing debris and promoting tissue healing. Behind the capacity of macrophages to react against environmental signals, there is a battery of membrane and cytosolic receptors that elicit intricate signaling networks. For a description of macrophage receptors involved in immune recognition see (Taylor et al. 2005).

The phenotypic characterization of macrophages developed in the 1980s, with the definition of macrophage-specific antigens such as F4/80 (EMR1) (Austyn and Gordon 1981) and CD68 (Micklem et al. 1989). Antibody staining made it possible to better detect macrophages in intact tissues and homogenates. From early experiments, it became clear that there was heterogeneity in the macrophage system when for example F4/80 showed differences in abundance within different macrophage populations and species (Hume et al. 1983). Also in the 1980s the Northern blot technique was established, where complementary specific probes are used to detect RNA levels (Alwine et al. 1977). The greater feasibility of nucleic acid probe design and synthesis versus antibody development gave advantage to RNA studies over protein studies.

With early Northern and Western blot studies the set of macrophage genes and proteins began to collectively emerge, which helped to clarify the role in function and disease for a variety of macrophage gene products. A few examples include identification of myeloid cells as sites of synthesis of coagulation factor XIII A1, a marker that is still used today to identify macrophages in tissue biopsies in the clinic (Weisberg et al. 1987), characterization of the synthesis and intracellular processing of human apolipoprotein E (APOE), a protein with great implications for atherosclerosis (Zannis et al. 1984), and characterization of the expression of the IL-1 family (Demczuk et al. 1987) and TNF (Fransen et al. 1985), two inflammatory mediators that form part of current anti-inflammatory targets in genetic and wide spectrum diseases such as rheumatoid arthritis.

The techniques to measure RNA levels have improved significantly in the last decades and today modern microarray and sequencing techniques are in place to compare and quantify a vast number of RNA molecules, with coverage approaching whole genome level (Mortazavi et al. 2008). Concomitantly our understanding of RNA species and their biological functions went beyond mRNA (messenger RNA), as we now know several non-coding RNA (ncRNA) species including microRNA (miRNA), long non-coding RNA (lncRNAs), and enhancer RNA (eRNA) which appear to operate a complex RNA-dependent regulation of the coding transcriptome (Mercer et al. 2009; Lieberman et al. 2013). With the increased resolution the term

transcriptome appeared. The transcriptome can today be broadly defined as the estimate of expression level and molecular identity of all RNA molecules produced in one or a population of cells. In macrophages, transcriptome studies have tremendous importance because, unlike T cells, macrophages do not undergo clonal expansion and instead achieve their remarkable phenotypic heterogeneity by remodeling their gene and protein contents.

28.3 The Basal Macrophage Transcriptome: Looking for an Identity Card

One of the key questions from a transcriptome point of view is "*What makes a macrophage a macrophage*?". The question has two angles: basic contents and contents specificity and involves defining the levels of mRNA, miRNA, and other RNAs. The goals are to identify novel macrophage functions and more specific markers. To provide estimates of mRNA abundance, sequencing tools are ideal since microarrays can be used to evaluate expression levels but preferably in a comparative manner. However, studies on macrophages using RNAseq are still limited, often dedicated to more specific matters and as for microarrays, establishing an expression cutoff value for rpkm (reads per kilo base per million) is of personal taste. A non-trivial element to consider is the macrophage species and the isolation and maturation protocols used in the studies in question. In this respect the knowledge and access to mouse ex vivo macrophages remains far more advanced than for humans, where the work is generally done in primary monocyte-derived macrophages and cell lines.

Recently we ranked the expression values obtained with microarrays of transcripts expressed in human in vitro primary macrophages matured with M-CSF, GM-CSF, human serum, and mouse ex vivo thioglycollate- and biogel-elicited peritoneal macrophages, and in vitro bone marrow-derived macrophages (Martinez et al. 2013). The best-expressed 1,000 genes across species were compared with a similar study of membrane and cytoplasmic proteomics. The comparison highlighted a signature of 87 genes and proteins conserved across species, also highly expressed in rat and fish macrophages. Soon after, Schultze and colleagues published a high resolution RNAseq comparison of in vitro primary macrophages matured with GM-CSF, and defined hitherto unrecognized differences between both maturation signals further discussed below (Beyer et al. 2012). By comparing our own microarray results with the RNAseq experiment of Schultze and colleagues, an RNAseq supported macrophage transcriptome begins to emerge, as 73 out of the 87 genes signature highly detected in human and mouse macrophages also appear amongst the best 1,000 expressed genes with sequencing methods. In Fig. 28.1 we briefly discuss an interactome analysis of the higher-ranking transcripts in both studies from a functional perspective.

The mere ranking of transcripts in isolated cells does not provide information about cell specificity. Two recent reports regarding macrophage specificity at

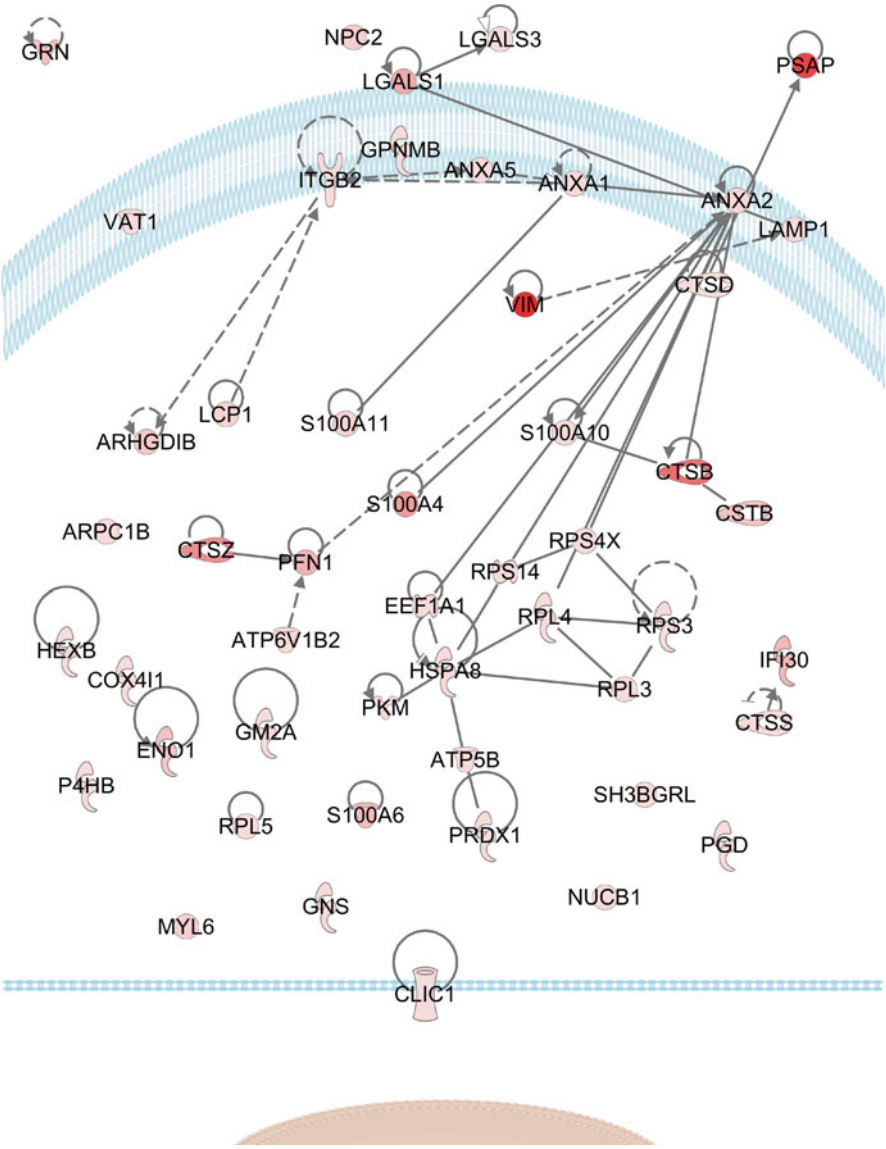

Fig. 28.1 Macrophage basal transcriptome. Using two different approaches with in vitro and ex vivo models we defined a set of 87 genes highly expressed in resting macrophages from human and mouse, detectable with microarray and proteomics (Martinez et al. 2013). Since microarrays are not designed for intra-array comparisons we wished to investigate how many of these genes are indeed detected as highly expressed in macrophages by RNA seq (Beyer et al. 2012). The comparison showed that 73 out of the 87 macrophage basal signature are also detected among the best-expressed genes by RNAseq; 50 of these genes were selected for interactome analysis. In the interactome appears a network involving Annexin A1 and A2 (ANXA1, ANXA2). Annexin A1 has potent anti-inflammatory effects (Perretti and D'Acquisto 2009), while Annexin A2 was proposed to promote inflammation (Swisher et al. 2007). Both Annexins appear to be involved in the

transcriptome level come from the Immunological Genome (ImmGen) Project, a collaborative scientific project dedicated to the definition of whole genome expression data for all characterized immune cells in the mouse (Miller et al. 2012; Gautier et al. 2012). The ImmGen dataset contains microarray results of several tissue macrophages, dendritic cells, and monocyte populations from C57BL/6J mice. Miller and colleagues focused on *bona fide* macrophage populations, such as peritoneal macrophages, red-pulp splenic macrophages, lung macrophages, and microglia. The authors noticed numerous differences between macrophage profiles, for example high CD11a and EPCAM levels on lung macrophages only, high VCAM-1 and CD31 in spleen macrophages, high CD93 and ICAM-2 in peritoneal macrophages, and high CX3CR1 and the lectin Siglec-H in microglia.

The heterogeneity in tissue macrophage populations made it difficult to find common macrophage signatures that distinguished them from DCs, let alone monocytes, and only 14 transcripts were expressed in all four macrophage populations but were not expressed in DCs. As for many macrophage studies, the authors had to compromise and extend their search for genes that were present in at least 50 % of their samples. This comparison identified already known macrophage mRNAs such as CD64 and TLRs. Other transcripts identified included the kinase FERT2. Conventional macrophage-associated genes such as MRC1 (which encodes the mannose receptor CD206), MARCO, and PPARG were not identified until two out of four macrophage populations were considered for the comparison. CD68, widely used to identify tissue macrophages, had similar expression in DCs and macrophages thus being excluded from the list. A total of 366 transcripts were absent or much lower in classical DCs than in macrophages, and only 39 of these transcripts were shared by the tissue macrophages compared.

Hume and colleagues provided a different perspective to the ImmGen study. As previously mentioned, Miller et al. focused on four macrophage populations, and excluded from the set of prototypical macrophages used for comparison monocytes, subcapsular sinus and medullary macrophages, elicited peritoneal macrophages, and salmonella-infected macrophages. Coexpression clustering with the network tool BioLayout Express3D showed that the tissue is determinant for the transcriptome of both cell types, as macrophages and DC clustered according to the tissue of origin and not to the cell type and some of the prototypical macrophages from different tissues appeared as different from each other as they are from DCs (Hume et al. 2013). In Fig. 28.2 we briefly discuss from a functional point of view an interactome of selected genes highly expressed in macrophages and not monocytes or DCs, deriving from a re-analysis of ImmGen data.

Fig. 28.1 (continued) phagocytosis of apoptotic cells (Fan et al. 2004). Linked to the annexins is Galectin-3 (LGALS3), a β-galactoside-binding lectin known to be abundantly expressed in macrophages (Liu et al. 1995), which plays a regulatory role in inflammation and fibrosis (Henderson and Sethi 2009), and is critically involved in IL-4 macrophage activation due to a positive feedback loop leading to sustained PI3K activation (MacKinnon et al. 2008). Genes involved in redox balance are over-represented and indirectly linked with the central cluster, with Peroxiredoxin 1 (PRDX1) an antioxidant and molecular chaperone, able to trigger TLR4 signaling and activation of NF-κB and secretion of TNF-α and IL-6 (Riddell et al. 2010)

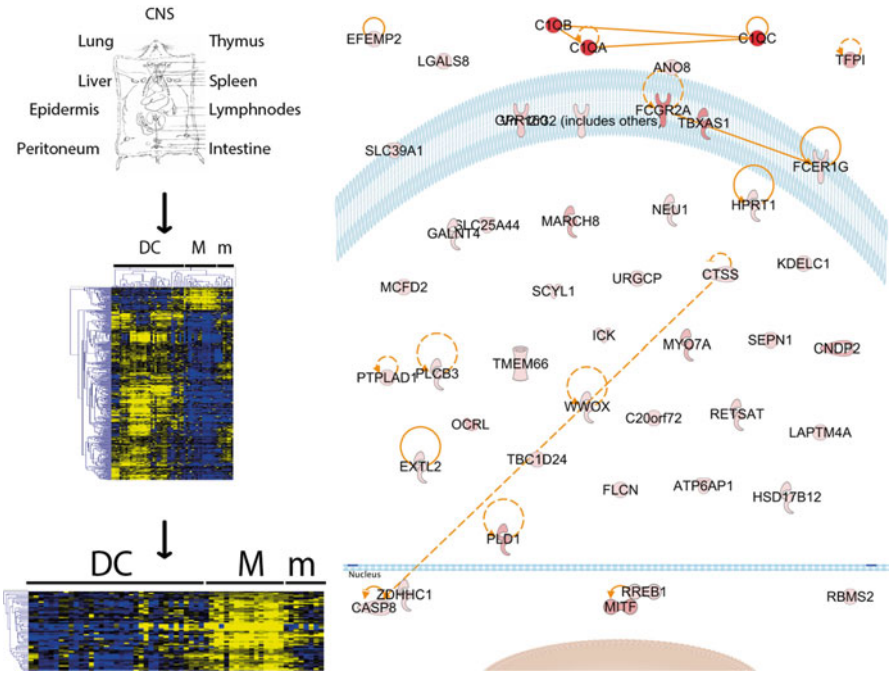

Fig. 28.2 Macrophage-specific transcriptome. By mining the ImmGen dataset we aimed to investigate the set of genes highly expressed in mouse macrophages and not DCs or monocytes. Monocytes and some macrophage populations were excluded in the comparisons published (Miller et al. 2012; Gautier et al. 2012). Briefly, an RMA matrix containing all dendritic cell populations, monocytes, and macrophage populations was downloaded from ImmGen, and statistically analyzed using ANOVA in TMEV. Changed genes were then organized according to their expression levels by hierarchical clustering using TMEV, and a sub-cluster containing 56 genes highly expressed in macrophages and not monocytes or DCs was selected for interactome analysis in IPA. In the interactome a network involving the C1q component can be seen. C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation and mediates antibody-dependent and independent immune functions important for phagocytosis, modulation of the humoral response, and canonical complement activation. FCGR2a and FCER1G, which appeared linked, are also highly expressed by macrophages vs DC and monocytes and are also involved in phagocytosis. CTSS is a lysosomal cysteine protease that may participate in the degradation of antigenic proteins to peptides for presentation on MHC class II molecules and appears linked indirectly with CASP8. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis but also in other macrophage functions such as response to TNF ligands and activation of other caspases that for example process IL-1β. Sepn1 appears highly expressed in macrophages and functions as protective mechanism caused by free radicals important for microbicidal functions

With these and other studies we begin to learn about global mRNA composition of macrophages and understand differences between members of the macrophage extended family beyond single markers. The functional interpretation of these profiles remains limited by current system biology and cell biology tools, but many functions remain to be identified if we consider the number of novel macrophage genes put forward and the functional relevance of the many, which we already knew of.

28.4 The Macrophage Transcriptome Within the Activation Paradigm

Studies about the transcriptome of activated macrophages are abundant and key findings have created paradigms regarding the role of macrophages. Macrophages undergo certain events in their "lifecycle" and we broadly organized them as maturation, priming, activation, and resolution (Fig. 28.3) (Gordon and Martinez 2010). Other definitions, e.g., M1, M2, M2a–c, exist for these types of activation but here we use the receptors, which leave no doubts about the pathway in question and avoids oversimplification (Mantovani et al. 2004). For each occasion the macrophage transcriptome and overall phenotype change, with consequences for the response to subsequent stimuli. The order used for this explanation is only of theoretical use since in tissues macrophages are exposed to stimuli in no specific order. However, simplifying signals and events in the macrophage is a useful strategy to understand the contribution of the different elements. The interplay between signals at different levels falls under the umbrella of the expanding concept of macrophage plasticity.

28.4.1 Maturation

During maturation, immature forms or macrophage precursors are prompted for survival, cytoplasm and membrane growth, increased adhesion, spreading, and phagocytic capacity. The main macrophage maturation cytokines in human and mouse are M-CSF and GM-CSF, but also IL-34 (Lin et al. 2008). However, although these three cytokines induce maturation, their effects and roles are different. M-CSF mutant mice show reduced levels of monocytes and selected macrophages, and osteopetrosis (Wiktor-Jedrzejczak et al. 1990). GM-CSF deficiency is less severe but drives nonfatal accumulation of alveolar material and vulnerability to pulmonary infections (Stanley et al. 1994). IL-34 absence affects Langerhans cell and microglia numbers (Wang et al. 2012). For signaling details and selected transcriptome signatures see Fig. 28.3.

The response to M-CSF in human monocytes was described at transcriptome level in a time course of monocyte to macrophage maturation (Martinez et al. 2006). The time course expanded over 7 days and we identified transient and stable gene clusters. Transient clusters were characterized by overrepresentation of cell cycle genes, e.g., cyclins (A2, B1, D1, E1) and downregulation of HLA members. Stable regulation gene clusters on the other hand mostly corresponded to membrane receptors such as TLR7 and immune response elements such as the complement C1QA/B/C units. A time course of GM-CSF was described by (Lehtonen et al. 2007). The authors characterized the transcriptome of macrophages matured in GM-CSF and compared them with DCs generated in parallel and found 342 genes upregulated and 194 down-regulated. Once again, transient and stable gene

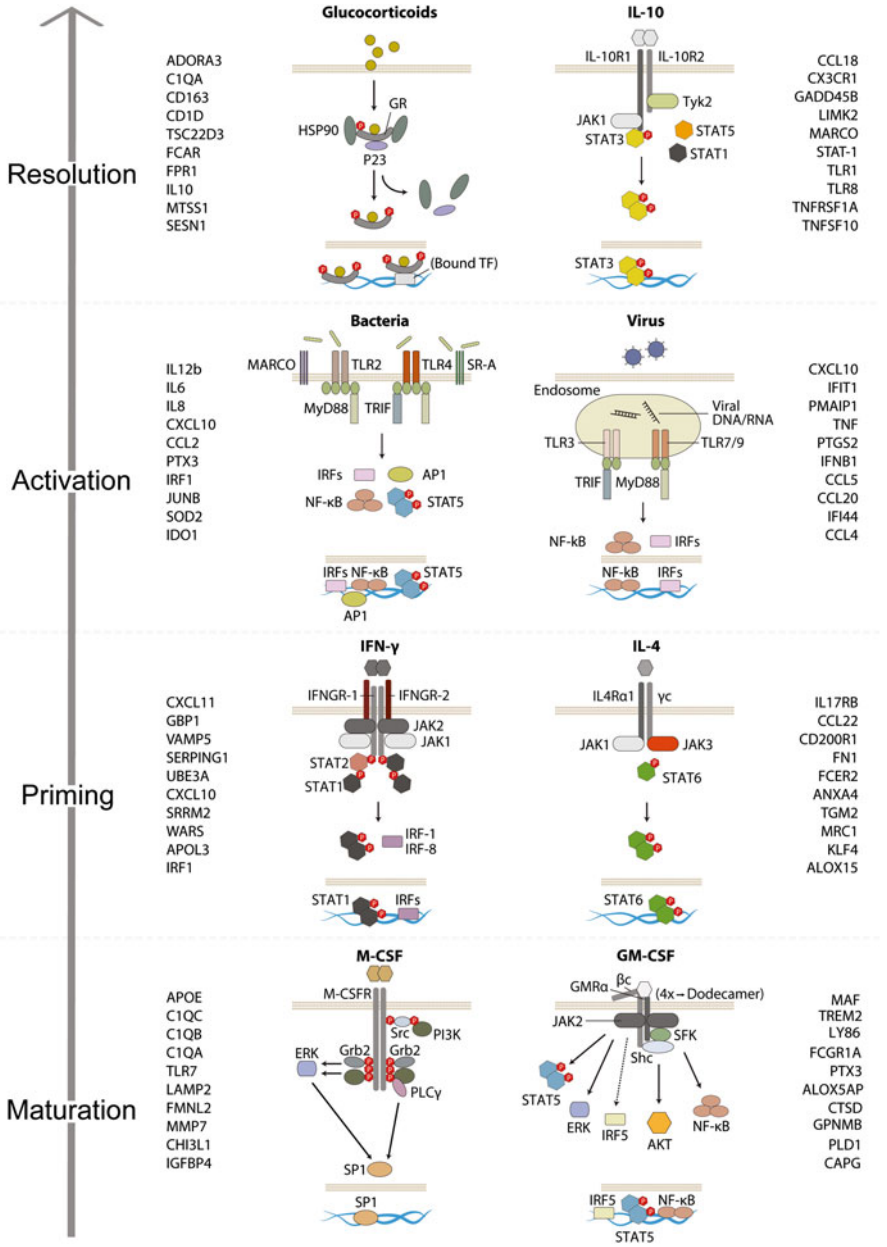

Fig. 28.3 Macrophage activation pathways and impact on the transcriptome. Receptors of the main activation pathways in macrophages are distinct while the signaling cascades share at times common elements and transcription factors. This makes it difficult to select transcriptome signatures specific for a given pathway. Here we revisit key signaling mediators in each pathway and provide selected regulated genes deriving from transcriptome studies discussed in the main text and referenced below. Selecting extensive multispecies signatures is difficult because of evolutionary

signatures appeared. The majority of the genes were regulated similarly in DCs, and only 76 genes were expressed at a higher level in macrophages. The macrophage-specific GM-CSF regulated genes included several known cell-surface molecules, for example CD14, FCGR1A and CD163, and new candidates such as BHLHB3, MCM2, MCM6, and NR1H3.

The majority of reports focus on the final differences between M-CSF and GM-CSF and early SAGE analyses already described differences between the models (Hashimoto et al. 1999). Hamilton and colleagues compared human and mouse macrophages matured in the presence of either cytokine and found 530 genes regulated in the same direction in both human and murine models. The genes represent 17 % of the total genes regulated. However, for the top 150 genes most differentially regulated in the same direction in all species, they detected only 11 genes in common

Fig. 28.3 (continued) divergence. We focused for this figure on human data; interspecies differences are beyond the scope of this chapter, but they are a key aspect to keep in mind. Maturation: The M-CSF receptor is a tyrosine kinase transmembrane receptor. M-CSF binding leads to receptor dimerization and autophosphorylation and activation of signaling pathways such as ERK, phosphatidylinositol 3-kinase, and phospholipase C and eventually Sp1 nuclear localization. The GM-CSF receptor is different since it does not possess intrinsic tyrosine kinase activity. Upon GM-CSF binding, the receptor forms a dodecamer structure (Hansen et al. 2008) and recruits JAK2 leading to the activation of STAT5, ERK, AKT as well as nuclear translocation of NF-kB and IRF5 (Krausgruber et al. 2011). Regulated genes were extracted from (Martinez et al. 2006) and (Lehtonen et al. 2007). Priming: The IFNGR-1 and IFNGR-2 chains form IFN-γ receptor. The receptor recruits Jak1 and Jak2 adaptors that activate STAT1, and interferon regulatory factors such as IRF-1 and IRF-8. Regulated genes were extracted from (Waddell et al. 2010). Three different receptor pairs recognize IL-4 and IL-13. IL-4Rα1 can pair with the common gamma chain (γc) enabling IL-4 binding and with the IL13Rα1 chain for IL-4 or IL-13 binding. In addition, IL-13 binds the IL13Rα2 chain, a controversial signaling receptor. Receptor binding of IL-4 activates JAK1 and JAK3. JAK activation leads to STAT6 activation and translocation. Other transcription factors are involved, such as c-Myc and IRF4. Regulated genes were extracted from (Martinez et al. 2013). Activation: Whole bacteria interact with TLRs leading to the recruitment of the signaling adaptor proteins MyD88 and TRIF. This causes the activation of a range of transcriptions factors such as IRF3, IRF5, STAT5, AP1, and NF-kB which initiates strong proinflammatory cytokine expression (O'Neill and Bowie 2007). The resulting expression profiles show great overlap with profiles of TLR4 and TLR2 stimulation. However, more receptors participate in the recognition of the whole pathogen including MARCO and SRA. Regulated genes were extracted from (Nau et al. 2002). Viruses are also recognized via TLRs. Viral proteins can activate TLR2 and TLR4, however, the full activation is mediated by the additional recognition of viral DNA or RNA in the endosome after endocytic uptake of the virus. As for bacteria, activation of different TLRs leads to the MyD88- and TRIF-mediated activation of transcription factors such as NF-kB and IRFs and subsequently to an initiation of a strong proinflammatory response. Regulated genes were extracted from (Lee et al. 2009). Resolution: Glucocorticoid hormones are secreted by the adrenal glands and further metabolized to different degrees of activity by cellular enzymes. Active glucocorticoids are lipophilic and diffuse through the membrane to bind the glucocorticoid receptor alpha, event with leads to nuclear translocation of the complex. GCR complex directly binds DNA to promote/repress gene transcription, or indirectly by regulatory protein–protein interaction with other transcription factors such as NF-kB or AP-1. Regulated genes were extracted from (Ehrchen et al. 2007). IL-10 binds the IL-10 receptor, a dimer of IL10R1 and IL10R2. Receptor autophosphorylation leads to activation of the transcription factor STAT3 and its binding mediates inhibition of proinflammatory cytokine expression. Regulated genes were extracted from (Park-Min et al. 2005)

(Lacey et al. 2012). The transcriptomes of M-CSF and IL-34 appear very similar in human macrophages (Barve et al. 2013). The gene expression profiles generated by IL-34 and M-CSF were compared at days 5 and 7 by Barve and colleagues, and 68–79 % of the transcripts regulated by the cytokines responded in a similar fashion. Among the genes differentially regulated the authors characterized CLEC4D, ECM1, CCR2, and SPPI.

28.4.2 Priming

In the presence of infection or damage, cells in the tissue release a variety of cytokines and danger signals that modulate the macrophage phenotype. In the case of acute events this subsides, but otherwise a lymphocyte response is involved. In the case of intracellular parasites, e.g., viral infection or bacteria, NK cells directly kill infected cells by the release of perforin and granzymes. In addition, they also secrete interferons (IFNs) and supported by T cells, and prime macrophages for enhanced phagocytosis and killing activity. Type-I IFNs include IFN-α, IFN-β, and IFN-ω, and the only type-II IFN is IFN-γ. In the opposed case of extracellular pathogens, such as nematodes, eosinophils and basophils are recruited and secrete IL-4 and IL-13, which induce alternative activation of macrophages. IFNs, IL-4 and IL-13 are thus produced by innate and adaptive immune cells, and link macrophages with lymphocyte and granulocyte activation programs.

In human macrophages, type-I and type-II IFN transcriptome profiles are scarce. A study by Waddell et al. compared the transcriptome of PBMCs and purified T, B, and NK cells and monocytes, to IFN-α, IFN-β, IFN-ω, IFN-γ, IL-12, and TNF (Waddell et al. 2010). The authors defined prototypical gene signatures to type-I IFNs. Interestingly, responses to IFN-γ and IL-12 were largely restricted to a subset of type-I IFN-inducible genes. Cell-type specific transcriptional programs were identified and monocytes had the greatest response to IFN-γ stimulation. The IFN conserved signature comprised cytokine receptors (CSF2RB, IL15RA, IL2RA, IL6R), cell activation markers (CD36, CD38, CD69, CD97), and a number of cell adhesion molecules (CD226, EVA1, ICAM1, ITGAL, ITGA4, ITGB7, LGALS3BP, MUC1, and SIAT1). The major mediators of IFN-γ induced signaling, STAT1, JAK2, and IRF1, and regulators CISH, NMI, PTPRC, PTPRO, SOCS1 were also changed. The temporal response of the monocyte population to IFN-γ treatment was analyzed and showed differences for functional categories, e.g., early induction of antigen presentation vs. late repression of chemotaxis.

IL-4 induces an alternative form of macrophage activation that leads to expression of selected cell-surface markers such as MRC1, increased endocytic capacity, trophic ability, and giant cell formation, all important for anti-nematode responses. In mice, the IL-4 transcriptome includes Arg1, Ym1, Fizz1, Mgl1/2, Tgm2, and Mrc1. TGM2 and MRC1 are useful markers for human IL-4 treated macrophages as well, but not Ym1 and Fizz1, which only exist in the mouse. Comparing human and mouse alternative activation signatures in a variety of models and time points we

recently defined a set of 17 transcripts consistently upregulated in alternatively activated macrophages in both species, and 19 down-regulated genes. As before a low stringency cut off (60 % of samples) was used due to differences between species. The genes upregulated include key enzymes, including TGM2, the cholesterol hydroxylase CH25H, and the prostaglandin-endoperoxide synthase PTGS1 (also COX1). Consistently modulated transcription factors and signaling modulators were also detected, e.g. IRF4, KLF4, CISH, and SOCS1 (Martinez et al. 2013). However, only TGM2 was consistently detected at mRNA and protein level in 100 % of the samples upon IL-4 treatment.

28.4.3 Activation

Monocytes and macrophages readily respond to pathogens and their MAMPs (microbial associated molecular patterns). Pathogen signals are often modeled and simplified by using MAMPs, e.g., TLR ligands. Both pathogens and ligands are strong modulators of the transcriptome per se, if not the strongest group. LPS or endotoxin is one of the best-studied macrophage signals and induces strong proinflammatory profiles that include cytokines, e.g. IL-12, TNF, IL-6, IL-1β, chemokines, e.g., CCL2, CXCL10, CXCL11, and antigen presentation molecules such as MHC members, co-stimulatory molecules, and antigen processing peptidases. The profiles are not limited to those genes but include thousands of transcripts. In human, studies with ligands other than LPS remain scarce. In mouse Shmulevich and colleagues studied the transcriptional responses of primary bone marrow-derived macrophages to six purified TLR agonists including LPS, Pam2CSK4, Pam3CSK4, CpG, poly I:C, and R848 (Ramsey et al. 2008). In addition to wild-type macrophages the authors studied TRIF, MyD88, ATF3, and CREM knockouts. A total of 95 distinct combinations of strain, stimulus, and time yielded 1,960 probe sets identified as differentially expressed, 44 % of which were found to be upregulated in LPS-stimulated wild-type macrophages, and 80 were transcription factors should read transcription factors (TF) genes were taken to constitute the set of potential regulators in the TLR-stimulated macrophage network. Time-lagged correlation (TLC) was then used to assess potential transcriptional regulatory interactions using the time-course expression data and strong associations were found for NF-κB, AP1, IRFs, STAT1, and EGR family members. Recently Schroder et al. investigated the diversification of LPS transcriptome in human and mouse macrophages and found that 24 % of orthologues identified were divergently regulated. Divergently regulated orthologues were enriched in cell-surface receptors, e.g., TLR6, IL-7Rα, and chemokines, e.g., CCL20, CXCL13. Functional consequences of divergent gene regulation were confirmed by showing LPS pretreatment boosts subsequent TLR6 responses in mouse but not human macrophages, in keeping with mouse-specific TLR6 induction (Schroder et al. 2012).

The transcriptome modifications in response to whole pathogens have been addressed in several studies, and generic signatures have been proposed for major

classes. Young and colleagues analyzed human macrophage gene expression upon exposure to different bacteria and found 977 genes significantly changed in response to one or more subtypes. Despite the diversity of the bacteria studied, a shared transcriptional response was elicited, consisting of 132 genes induced and 59 repressed. The genes changed in common contained proinflammatory genes, including many cytokines and chemokines previously mentioned. However, the majority of the transcriptome elicited involved receptors, signaling molecules, transcription factors, adhesion molecules, genes involved in tissue remodeling, enzymes, and anti-apoptotic molecules. For example macrophages upregulated many of the NF-κB pathway members receptors for chemokines and cytokines such as CCR6, CCR7, IL-7R, and IL-15Rα (Nau et al. 2002). Importantly the authors could ascribe major parts of the pathogen profiles to TLR ligands such as LPS, muramyl dipeptide, and lipoteichoic acid.

Viral responses in macrophages are also largely dependent on TLR recognition. Similar to the recognition of bacteria this leads to activation of IRFs and NF-κB causing a strong proinflammatory response. Virus effects are not well described from a transcriptome point of view in macrophages, although we can hypothesize that as for bacteria there are conserved signatures and virus specific signatures. Viral proteins are recognized by cell membrane-bound TLR2 and TLR4, but also other receptors. After uptake, viral DNA or RNA are recognized by endosomal TLRs, e.g. TLR3, TLR7, TLR8, and TLR9. Influenza has deserved great attention due to its pathogenicity, and is one of the few viruses that have been studied in macrophages (Lee et al. 2009). A comparison between the highly pathogenic H5N1 versus to the seasonal H1N1 showed conserved responses to both viruses, although the number of genes and regulation changes correlated with the strain virulence. H5N1 and H1N1 induced type-I IFN, and a variety of IFN regulated genes such as 2′-5′-oligoadenylate synthetases, OASL and OAS2, guanylate binding proteins GBP1, GBP4, GBP5, and GBP7, MX1, IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), and the prototypic inflammatory cytokines TNF, IL-1β, CXCL9, CXCL11, CCL5, and 8. Other viruses require further investigation.

Macrophage responses to pathogens or their ligands are strong, but the macrophage can go a step further when it comes to activation. Mackaness and colleagues described that macrophages acquired enhanced microbicidal capacity in the presence of lymphocyte products, specifically IFN-γ (Nathan et al. 1983; Mackaness 1962). Today the sources of IFNs are known to vary from innate to adaptive immune cells as the immune response evolves, a fact that is also true for other cytokines such as IL-4, IL-13, and TGF-β. The interaction between cytokines other than IFN-γ and pathogens remain ill defined. In fact we know little about the possible interactions between IL-4 and these pathways or functions that could be enhanced by the combination. The timing of pathogen response and cytokine response will vary for different macrophages, *ergo* it is difficult to indicate a clear hierarchy in these events.

28.4.4 Resolution

Macrophages contribute to the resolution of the inflammatory response and acquire anti-inflammatory phenotypes with decreased secretion of conventional inflammatory cytokines such as TNF, IL-6, or IL-1β. The process is not a real deactivation but another form of activation that promotes removal of cellular debris and induces tissue repair. The main cytokine with this role is IL-10. Seminal studies showed IL-10 as Th2 product able to inhibit Th1 cells (Fiorentino et al. 1989). IL-10-deficient mice develop inflammatory bowel disease following colonization of the gut with resident enteric bacteria (Sellon et al. 1998) and show exaggerated inflammatory responses to parasites (Gazzinelli et al. 1996). IL-10 is produced by virtually all leukocytes. In macrophages, IL-10 is elicited in response to TLR activation, glucocorticoids, and C-type lectin signaling, e.g., DC-SIGN and dectin 1 ligation by pathogens. The macrophage transcriptome upon IL-10 stimulation remains ill defined, but long and short-term monocyte profiles have been described for humans (Park-Min et al. 2005). Park et al. found that 2 days IL-10 exposure induced expression of 133 genes, whereas 7 days of IL-10 exposure induced expression of 250 genes. The receptors for the Fc region of immunoglobulins, FCGRI, II, and III, which mediate phagocytosis, were strongly induced by IL-10 in a time-dependent manner, as well as the chemoattractants CXCL13, CXCL4, and pattern recognition receptors FPR1, TLR1, TLR8, and MARCO.

Glucocorticoids also induce macrophage pro-resolution programs. Expression analyses of monocytes stimulated with the glucocorticoid fluticasone propionate showed a shift in the phenotype with 102 genes significantly upregulated and 45 down-regulated genes after 18 h. The profiles include induction of C1QA, DSIPI, MRC1, THBS1, IL-10, IL1R2, CD163 (Ehrchen et al. 2007). Using in silico functional analysis the authors predicted that key monocyte features such as adherence, cell spreading, phagocytosis, and apoptosis would be affected, and confirmed these observations at the functional level. For example, among the genes upregulated FPR1 conferred increased migration toward FMLP while increasing the response to anti-inflammatory lipids.

TGF-β, another cytokine with deactivation properties, has been studied from a transcriptome point of view in humans (Gratchev et al. 2008). Analysis of the expression profiles on IL-4 or IL-4 + dexamethasone-treated macrophages showed that TGF-β was active only in the presence of glucocorticoids. The kinetics of TGF-β induced gene expression revealed two groups of genes, a group of 44 genes at 3 h and a group of 90 genes at 24 h. The overlap consisted of 23 genes. TGF-β genes were involved in immune response and included receptors such as IL18R1, IL17RB, CXCR4, transcription modulators such as FOS, JUNB, and LTA4H involved in leukotriene synthesis. The profile was also enriched in lipid binding, transport, and processing, including the receptor for oxidized low density lipoprotein OLR1 and several apolipoproteins, e.g., APOE, APOC1, APOC2.

Activation transcriptome studies in vitro and in vivo have helped to understand the versatility of macrophages and begin to shed light on the specificity of stimuli,

coincidences between species, and mechanistic and kinetic insights into the regulatory pathways leading to gene expression. All resolution events in the macrophage are accompanied by gene expression changes, which challenge the view of certain stimuli such as glucocorticoids and IL-10 as mere deactivators.

28.5 The Macrophage Transcriptome Outside Messenger RNA

One striking observation revealed by transcriptome studies is that even if around 90 % of DNA is transcribed, only 1–2 % of the transcriptional output encodes for proteins (Consortium 2004). This has led researchers to consider the vast majority of ncRNAs as intrinsic part of the global transcriptome of a cell, further adding complexity and potentially new layers of genomic expression. NcRNAs represent at the moment an emerging field in macrophage studies. Thus this section will specifically focus on three classes of ncRNAs, namely miRNAs, eRNAs, and Large intergenic RNAs (lincRNAs) and provide examples of how genome-wide analysis of these ncRNAs add to our comprehension of macrophage identity and function.

28.5.1 miRNAs

The best-characterized class of ncRNAs are microRNAs (miRNAs). MiRNAs are evolutionary conserved small RNAs that finely tune gene networks via interference mechanisms. MiRNAs exert their silencing action within a multiproteic complex called RISC (RNA-induced silencing complex), by binding specific sequences within the 3'UTR of their target RNAs or, as more recently described, in the coding region of their target mRNAs (Tay et al. 2008). MiRNAs are found either "intragenic" or "intergenic" and are transcribed as long precursors by RNA Pol II (pre-miRNAs) and then processed in two steps, nuclear and cytosolic, to give rise in the cytoplasm to mature 21–24 nucleotide long miRNAs.

Hitherto the basal miRNome of macrophages has not been addressed, but the information begins to emerge. By using RT-PCR-based Taqman Low Density Assays (TLDA), Graff and colleagues studied the regulation of selected miRNAs by activation stimuli discussed below (Graff et al. 2012). The authors found 249 basal miRNAs expressed in human macrophages, irrespective of the activation status. A more recent study described the first RNAseq miRNome of human macrophages differentiated with M-CSF (Swaminathan et al. 2013). The RNAseq data reveals a set of 701 basal miRNAs present in macrophages. Ranking these, a discrete number of already "macrophage-associated" miRNAs can be identified, some of them well characterized such as miR-21, miR-146a/b, miR-142, miR-155, miR-125a, and miR-223. For instance miR-142, better described for taking part in dendritic cell homeostasis (Mildner et al. 2013), has recently been associated with

M-CSF-dependent monocyte to macrophage maturation (Lagrange et al. 2013). MiR-146a is differentially expressed among human and murine monocyte subsets and therefore implicated in monocyte heterogeneity by mechanistically targeting the non-canonical NF-κB/Rel family (Etzrodt et al. 2012). MiR-21 and miR-155 are well-described LPS-induced miRNAs and promote or sustain TLR4-induced proinflammatory responses.

Graff et al. focused on defining the miRNome of activated macrophages. The authors included in their study resting macrophages exposed or not to LPS + IFN-γ, IL-4, IgG + LPS and finally TGF-β1, and found 13 miRNAs significantly regulated. The most important changes in expression levels when compared to resting macrophages were observed for miR-125a-3p, induced by the combination of LPS + IFN-γ, miR-193b induced by IL-4, and miR-27a induced by combination of IgG + LPS. MiR-155 (Arranz et al. 2012) and miR-378a-3p (Ruckerl et al. 2012), two established LPS and IL-4 responsive mouse miRNAs, were not upregulated suggesting that as for mRNAs, interspecies and model differences exist for miRNAs expression. Swaminathan et al. described seven new miRNAs in their study, 4 of them differentially modulated by IL-27. Finally, miR-187 and miR-146b have been recently identified as IL-10-dependent miRNAs in macrophages, and have been shown to operate a preferential targeting of the TLR signaling pathway (Rossato et al. 2012; Curtale et al. 2013), suggesting their involvement in the resolution activity of IL-10. These early studies begin to shed light into this ulterior level of macrophage identity and activation.

28.5.2 eRNAs

Recent RNA-seq and RNA Pol II ChIP-seq studies describe another class of ncRNAs, eRNAs. These are short RNA species of size ranging from 0.1 to 9 kb transcribed from enhancer regions of genomic DNA (Kaikkonen et al. 2011). eRNAs are short lived and produced from extragenic regions enriched for enhancer-associated histone marks such as monomethylation of lysine 4 of histone H3 (H3K4me1) and low trimethylation of lysine 4 of histone (H3K4me3) combined with the presence of RNA Pol II and the transcriptional co-activator p300. Little is known about eRNAs in human macrophages. In mouse macrophages stimulated with LPS and IFN-γ, the expression of eRNAs has been shown to precede that of the adjacent protein-coding gene, supporting a functional role. De Santa and colleagues showed that a large fraction of extragenic RNA Pol II chromatin immunoprecipitates (69 % of RNA Pol II peaks) was associated with enhancer regions. They also show that macrophage activation with LPS and IFN-γ changed the transcriptional output of these regions (De Santa et al. 2010); some remain transcribed (20 %) while other were induced (32 %) or repressed (48 %). Interestingly, the three classes of enhancer were differentially enriched for transcription factor binding sites and the inducible group was associated with NF-κB and IRFs. Functionality was demonstrated for the enhancer region upstream of the Ccl5 gene, controlled by NF-κB and IRF3.

Nuclear receptors control eRNAs expression in macrophages (Lam et al. 2013). A recent genome-wide Rev-Erbα and Rev-Erbβ ChIP-Seq analysis by Lam and colleagues showed that 70 % of Rev-Erbs peaks mapped with enhancers along the genome. Analysis of selected genomic regions containing Rev-Erbs showed that for example the 5 kb Mmp9 and 28 kb Cx3cr1 enhancers indeed exhibited transcriptional activity. Gain of function experiments for Rev-Erbα led to decrease eRNAs expression while the loss of function led to derepression of eRNAs. In a mouse sterile peritonitis model Mmp9 eRNA-specific siRNA led to reduced expression of the transcript.

28.5.3 lincRNAs

lincRNAs are a subclass of lncRNAs found in intergenic genomic regions. They are typically longer than 200 nucleotides and can display a size of several kilobases. Seminal work by Guttman and colleagues described this class of ncRNAs as being marked by trimethylation of lysine 4 of histone H3 (H3K4me3) at their promoter region and by trimethylation of lysine 36 of histone H3 (H3K36me3) along their transcribed region, which are both characteristic marks of active gene transcription by RNA Pol II (Guttman et al. 2009). Of note, lincRNAs transcripts exhibit generally a good conservation among species, a characteristic also found for their promoter regions (to a similar degree of promoters of protein-coding genes). LincRNAs were furthermore found to be more tissue/cell specific than protein-coding RNAs.

Although little is known about the macrophage lincRNA repertoire, or how these lincRNAs impact on macrophage biology, Subramaniam and colleagues recently studied lincRNAs in resting and LPS-activated macrophages in a mouse model (Garmire et al. 2011). This profiling study showed that 378 lincRNAs are expressed by resting murine macrophages, and 189 were expressed in response to LPS stimulation. Among the 189 lincRNAs responsive to LPS treatment, 45 were down-regulated and 126 were upregulated compared to basal condition. Functional pathway analysis of genes neighboring the lincRNAs showed that “resting” lincRNA-associated genes were associated to metabolic processes whereas genes associated to lincRNAs expressed upon LPS stimulation were found to be enriched for immune system processes and pathways related to cytokine–cytokine receptor interaction.

28.6 The Macrophage Transcriptome in Disease

Aside from transcriptome studies about pathogen response discussed above, great interest lies in studying macrophages in chronic diseases such as COPD, asthma, RA, and malignancies, to mention a few. The access to tissue and cell isolation methods for humans are still scarce compared with mouse, however macrophages from several diseases have been profiled in both species. The majority of chronic

diseases are qualified by their T cell repertoire, making them Th2 diseases, Th1 diseases, Th17 diseases. However, until now no report has shown "pure" stimuli signatures in vivo, and instead disease specific profiles appear to be mixed at transcriptome level. For example when matching studies of alveolar macrophages in asthma, a predominantly Th2 disease, the profile is far from being that of macrophages stimulated in vitro with IL-4 or IL-13. In some diseases such as atherosclerosis, macrophages have profiles that do not fit any clear cytokine or lipid mediated transcriptome pattern in vitro (Feig et al. 2012). In this section we wanted to focus on one aspect emerging from transcriptome studies, i.e., macrophage activation in disease does not consist of uniform programs that can always be matched to in vitro activation programs.

Macrophages in disease can carry mixed activation programs. Tumors are populated by macrophages (TAM) that can promote tumor progression to malignancy and increase metastatic potential through the stimulation of tumor cell migration, invasion, and angiogenesis, an effect associated with alternative activation. Pettersen et al evaluated expression of phosphorylated STAT6 in human cutaneous squamous cell carcinoma TAM based on Porta's seminal work (Sica et al. 2006), and increasing association of Th2 cells with the tumor microenvironment (de Oliveira et al. 2009; Todaro et al. 2008). STAT6p co-localization with CD163 was abundant in the inflammatory infiltrate associated with SCC compared to normal skin. In addition the macrophages also expressed established IL-4 markers such as CD209 (DC-SIGN), CCL17, and CCL18 supporting the presence of IL-4 activation in TAMs. The authors went further and used gene set enrichment analysis using classical and alternative activation signatures. The enrichment analysis showed that instead of alternative programs, TAM profiles were enriched for classical activation markers. TAM in SCC strongly expressed IFNγR1 and IFNγR2 compared to normal skin, and IFN regulated genes such as STAT1, phosphorylated STAT1, Mx-1 were upregulated. Interestingly at tissue level the authors confirmed the presence of macrophages expressing alternatively activation markers only such as CD209 and CCL18, macrophages that expressed classical activation markers only such as phosphorylated STAT1, IL-23p19, IL-12/IL-23p40, and CD127, and macrophages that were bi-activated (Pettersen et al. 2011).

In addition to having mixed phenotypes, in lesions macrophage subsets change depending on the time course of the chronic illness disease in question. Spinal Cord Macrophages dominate sites of CNS injury promoting injury or repair. Using cDNA microarrays Kigerl and colleagues analyzed temporal changes in the expression profiles for genes associated with classical or alternative macrophages between 1 and 28 days after injury (Kigerl et al. 2009). In the injured spinal cord, classical and alternative signatures were rapidly induced. Alternative gene programmes were transient and returned to preinjury levels after 7 days, while classical activation gene patterns were maintained for up to 1 month after injury. Thus in addition to the heterogeneity found in a specific moment, the temporal regulation of activation and development of specific macrophage subsets is an important feature of disease.

Macrophage signatures in disease not always fit the predictions. COPD is a lung disease linked with tobacco smoking. The disease is often conceived as immune antithesis of asthma. As such, a classic rather than alternative proinflammatory

profile would be expected in transcriptome analysis. Woodruff and colleagues studied the response to cigarette smoke of alveolar macrophages in 70 subjects including healthy never-smokers with normal lung function, healthy smokers with normal lung function, and COPD smokers (Woodruff et al. 2005). The authors found 72 genes increased and 38 genes decreased in smokers compared with healthy non-smoking control subjects. The gene most highly induced in smokers was CYP1B1, a cytochrome P450 enzyme and MMP-12 also implicated in several mouse models of emphysema. Unexpectedly, smoking down-regulated T cell-recruiting chemokines such as CXCL11, CXCL9, CCL5, CCL4, IFN signaling molecules such as GBP1, 2, 4, and 5 and other inflammatory markers such as CD69, CD80, IL-1β, IL-18, TNFSF1, and C3aR. Alternative activation genes were more expressed in healthy smokers but a uniform signature was not found, e.g., MRC1, DECTIN1, and CD163 were not significantly modulated by smoking. Comparison with a dataset of asthmatic patients showed that only ten genes were differentially expressed between subjects with stable asthma and healthy nonsmokers, and none of these were among the genes that were differentially expressed in smokers. Overall, the panel of smoking-induced probe sets in alveolar macrophages represented a mixture of different subtypes of macrophage activation with up-regulation of genes related to IL-4, glucocorticoid, IL-10 as well as IFN-γ. These results were confirmed in an independent transcriptional profiling study of alveolar macrophages of 24 healthy nonsmokers, 34 healthy smokers, and 12 COPD smokers (Shaykhiev et al. 2009). The second study showed that healthy smokers exhibited a unique polarization pattern characterized by substantial suppression of classical activation-related inflammatory/immune genes and induction of genes associated with various alternative activation programs relevant to tissue remodeling and immunoregulation. Such reciprocal changes progressed with the development of COPD, with classical activation-related gene expression being most dramatically down-regulated. Again the progressively down-regulated genes included the chemokines CXCL9, CXCL10, CXCL11, and CCL5. Progressive activation of alternative-related program was characterized by induction of tissue remodeling and immunoregulatory genes such as MMP2, MMP7, and adenosine A3 receptor (ADORA3). The data provide transcriptome-based evidence that contrary to expectations, COPD pathogenesis involves deactivating classical activation programs and partially inducing alternative activation gene signatures.

In summary the model where predominant T cell profiles determine the macrophage gene signatures and induce homogeneous response, is challenged by observations where macrophages programs are a mosaic of different activation signatures, exclusive of each other or not, which develop with a certain degree of independence as lesions progress.

28.7 Conclusions

Investigating the macrophage transcriptome is important to understand novel functions and define upstream regulators of pharmacological use. In this chapter we discussed selected manuscripts dealing with the macrophage transcriptome: basal

and specific, within the activation paradigm and the transcriptome in disease. A few aspects emerge from these and other studies.

The basal and specific macrophage transcriptome is composed of many genes in their majority linked with immune recognition, phagocytosis, redox pathways, and the lysosomes. A considerable number of the highly expressed genes are shared within species, and at cell level with other members of the myeloid system such as monocytes and dendritic cells. The genes macrophage specific, and thus the differences between dendritic cells and monocytes require further investigation, especially in humans. The similarities among DCs and macrophages and monocytes may be greater than expected at transcriptome level, and the few differences reported seem to involve the phagocytic machinery and metabolic processes. The transcriptome differences between tissue macrophages seem considerable supporting a dominant role of the tissue environment in macrophage and DC properties and gene signatures.

The macrophage activation paradigm is gaining clarity and transcriptome studies show that there are similarities, as well as differences, between maturation, priming, activation, and resolution stimuli. The signatures of activation although informative need reinterpretation considering the overlap between stimuli, and the little we know about the majority of genes detected. Biased analysis and interpretation also can hinder the meaning of different signatures elicited by activating stimuli. In the chapter we have reported the number of genes that changed in different studies to render an idea of how strong are the different stimuli and events leading to acquisition of mature or activated phenotypes, but also the divergence between models and species. So far TLR ligands are the strongest category of stimuli followed by maturation and priming stimuli. TLR ligand programs seem transient while for maturation and priming stimuli the length of stimulation increases the number of genes, although in almost every process the signatures are complex and formed by transient and stable waves.

In disease, the signatures seem complex and macrophages with different, mixed or not, phenotypes have been reported. These are findings that shift the model of macrophage activation in disease, showing greater heterogeneity than expected, and could influence the attempts to treat and revert macrophage activation as therapeutic tool. The regulation of these important macrophage signatures is mediated by specific receptors, pathways with discrete overlap, but importantly by signatures of ncRNA that form part of the macrophage transcriptome. ncRNAs are important for the development and control of activation in macrophages and are modulated as much as mRNA in response to life events in the macrophage.

In all, the macrophage transcriptome contains vast information and provides a new view of the cell, which explains the exceptional macrophage functional versatility and provides unique tools to understand disease and conceive new macrophage oriented therapies.

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Appendix A. Gene Symbols in the Chapter

APOC1	Apolipoprotein C-I
APOC2	Apolipoprotein C-II
APOE	Apolipoprotein E
Arg1	Arginase 1
ATF3	Activating transcription factor 3
BHLHE41	Basic helix-loop-helix family, member e41
C1QA	Complement component 1, q subcomponent, A chain
C3AR1	Complement component 3a receptor 1
CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
CCL2	Chemokine (C-C motif) ligand 2
CCL20	Chemokine (C-C motif) ligand 20
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CCNA2	Cyclin A2
CCNA2	Cyclin A2
CCNB1	Cyclin B1
CCND1	Cyclin D1
CCNE1	Cyclin E1
CCR2	Chemokine (C-C motif) receptor 2
CCR6	Chemokine (C-C motif) receptor 6
CCR7	Chemokine (C-C motif) receptor 7
CD14	CD14 molecule
CD163	CD163 molecule
CD1a	CD1a molecule
CD200R1	CD200 receptor 1
CD209	CD209 molecule
CD226	CD226 molecule
CD36	CD36 molecule
CD38	CD38 molecule
CD68	CD68 molecule
CD69	CD69 molecule
CD80	CD80 molecule
CD93	CD93 molecule
CD97	CD97 molecule
CH25H	Cholesterol 25-hydroxylase
Chi3l3	Chitinase 3-like 3
CISH	Cytokine inducible SH2-containing protein
Clec10a	C-type lectin domain family 10, member A
CLEC4D	C-type lectin domain family 4, member D
CLEC7A	C-type lectin domain family 7, member A
CREM	cAMP responsive element modulator
CSF1	Colony stimulating factor 1 (macrophage)

CSF2	Colony stimulating factor 2 (granulocyte–macrophage)
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte–macrophage)
CX3CR1	Chemokine (C-X3-C motif) receptor 1
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL11	Chemokine (C-X-C motif) ligand 11
CXCL13	Chemokine (C-X-C motif) ligand 13
CXCL4	Chemokine (C-X-C motif) ligand 4
CXCL9	Chemokine (C-X-C motif) ligand 9
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
Cyr61	Cysteine-rich, angiogenic inducer, 61
ECM1	Extracellular matrix protein 1
EMR1	egf-like module containing, mucin-like, hormone receptor-like 1
EPCAM	Epithelial cell adhesion molecule
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
FERT2	fer (fms/fps related) protein kinase, testis specific 2
FOS	FBJ murine osteosarcoma viral oncogene homolog
FPR1	Formyl peptide receptor 1
ICAM1	Intercellular adhesion molecule 1
ICAM2	Intercellular adhesion molecule 2
IFNA1	Interferon, alpha 1
IFNB1	Interferon, beta 1, fibroblast
IFNG	Interferon, gamma
IFNW1	Interferon, omega 1
IL10	Interleukin 10
IL12	Interleukin 12
IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
IL13	Interleukin 13
IL15RA	Interleukin 15 receptor, alpha
IL17RB	Interleukin 17 receptor B
IL18	Interleukin 18
IL18R1	Interleukin 18 receptor 1
IL1B	Interleukin 1, beta
IL1R2	Interleukin 1 receptor, type-II
IL23A	Interleukin 23, alpha subunit p19
IL27	Interleukin 27
IL2RA	Interleukin 2 receptor, alpha
IL34	Interleukin 34
IL4	Interleukin 4
IL6R	Interleukin 6 receptor
IL7R	Interleukin 7 receptor
IRF1	Interferon regulatory factor 1
Irf3	Interferon regulatory factor 3
IRF4	Interferon regulatory factor 4

ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
ITGAL	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)
ITGB7	Integrin, beta 7
JAK2	Janus kinase 2
JUNB	Jun B proto-oncogene
KLF4	Kruppel-like factor 4 (gut)
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein
LTA	Lymphotoxin alpha
LTA4H	Leukotriene A4 hydrolase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MARCO	Macrophage receptor with collagenous structure
MCM2	Minichromosome maintenance complex component 2
MCM6	Minichromosome maintenance complex component 6
Mgl2	Macrophage galactose *N*-acetyl-galactosamine specific lectin 2
MMP12	Matrix metallopeptidase 12 (macrophage elastase)
Mmp9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)
MPZL2	Myelin protein zero-like 2
MRC1	Mannose receptor, C type 1
Msr1	Macrophage scavenger receptor 1
MUC1	Mucin 1, cell-surface associated
MyD88	Myeloid differentiation primary response 88
MYO1B	Myosin IB
NCS1	Neuronal calcium sensor 1
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NMI	N-myc (and STAT) interactor
NR1D1	Nuclear receptor subfamily 1, group D, member 1
NR1D2	Nuclear receptor subfamily 1, group D, member 2
NR1H3	Nuclear receptor subfamily 1, group H, member 3
OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1
PECAM1	Platelet/endothelial cell adhesion molecule 1
PPARG	Peroxisome proliferator-activated receptor gamma
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
PTPRC	Protein tyrosine phosphatase, receptor type, C
PTPRO	Protein tyrosine phosphatase, receptor type, O
RETNLB	Resistin like beta
SIAT1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1
Siglec-H	Sialic acid binding Ig-like lectin H
SOCS1	Suppressor of cytokine signaling 1
ST14	Suppression of tumorigenicity 14 (colon carcinoma)
STAT1	Signal transducer and activator of transcription 1, 91 kDa
TGFB1	Transforming growth factor, beta 1

Tgm2	Transglutaminase 2
TLR1	Toll-like receptor 1
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TNF	Tumor necrosis factor
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B
TRIF	Toll-like receptor adaptor molecule 1
TSC22D3	TSC22 domain family, member 3
VCAM1	Vascular cell adhesion molecule 1
Wnt5b	Wingless-type MMTV integration site family, member 5B

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Chapter 29
Omics Approaches to Macrophage Biology

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29.1 Introduction

Macrophages, terminally differentiated myeloid cells, are innate immune cells expressing a plethora of membrane bound and cytosolic receptors. Through these receptors, macrophages respond to a number of natural, altered-self, and microbial products such as lipopolysaccharide (LPS) (Taylor et al. 2005). To study their role in immune response and resolution, macrophages are exposed to a variety of ligands and samples are collected. Measurements of gene transcript, protein, and metabolite

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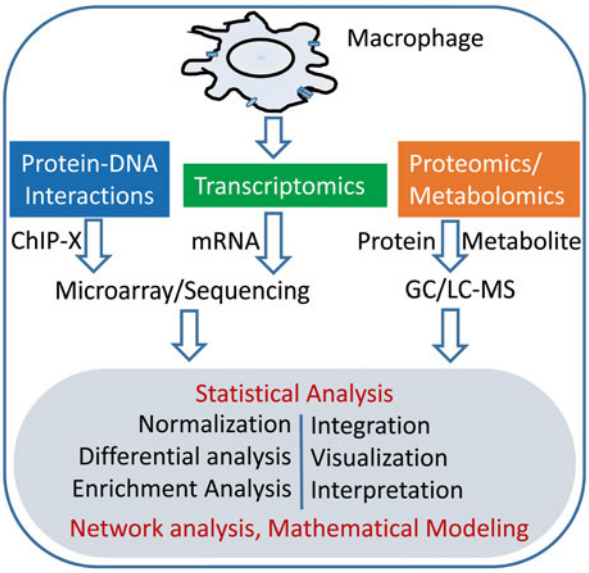

Fig. 29.1 Overview of the omics data and bioinformatics approaches to study macrophages

levels describe the functional state of macrophages and can provide mechanistic insight into their functions. The advent of HTP technologies such as Microarray, Sequencing (Seq), and Mass Spectrometry (MS) has revolutionized biomedical science by enabling the measurement of thousands of genes, proteins, and metabolites from a single sample (Mortazavi et al. 2008; Aebersold and Mann 2003; Dennis et al. 2010) or in a single cell (Navin et al. 2011; Shi et al. 2012). However, the data produced by these HTP technologies are subject to the "curse" of high dimensionality resulting from a large number of measured variables compared to the number of conditions. Further, this data are also subject to low signal-to-noise levels because of the sensitivity of the technology as well as the large number of genes, proteins, or metabolites present in the sample. Various bioinformatics approaches are used to handle such issues. In this chapter, we succinctly describe different types of omics data followed by methods used for their analysis (Fig. 29.1).

29.2 Omics Data and Methods

29.2.1 Transcriptomics

Hybridization or sequencing (Seq) based techniques are now routinely used for genome-wide transcriptomic measurements. Microarray uses the hybridization technique and produces intensity profiles for known genes, whereas RNA-Seq utilizes the basic principles of Sanger sequencing and reports millions of nucleotide sequences as short sequence reads (SSR). These SSR are aligned to a reference

genome using tools such as Burrows–Wheeler Aligner (BWA) (Li and Durbin 2009), Bowtie (Langmead et al. 2009), and Omicsoft Sequence Aligner (OSA) (Hu et al. 2012). For further analysis, genome mapping details may be exported in Sequence Alignment/Map (SAM), compressed binary version of the SAM (BAM) or Browser Extensible Data (BED) file formats (Quinlan and Hall 2010; Li et al. 2009), and visualized directly on the UCSC Genome Browser (Karolchik et al. 2003). Mapping of these SSR provides the count for a particular mRNA species in the sample. RNA-Seq is not limited to quantification of known gene transcripts, but also permits de novo assembly of the transcriptome and identification of novel mRNAs, intron-exon junctions, and single nucleotide polymorphisms (SNP) (Wang et al. 2009). RNA-Seq is also used for sequencing of small RNAs, including microRNAs (miR). Small RNA-Seq utilizes gel electrophoresis for small RNA enrichment followed by the RNA-Seq method (Hafner et al. 2008; Lu et al. 2007). Data are commonly normalized using either trim mean, global, Lowess, or quantile normalization to eliminate systemic and procedural noise (Quackenbush 2002; Bolstad et al. 2003). Because of the additional advantages and continuously decreasing cost of RNA-Seq, it is evolving as the preferred method for transcriptomic measurements.

In Microarray or RNA-Seq studies, regulation of genome-wide transcriptional activity is assessed by comparing one or more experimental conditions with a control condition. Differentially regulated transcripts (genes) can be naïvely identified using the fold change approach. However, due to sensitivity of the instruments at low probe intensity in microarrays or due to stochastic effects at low copy number in RNA-Seq, the variance structure of microarrays or RNA-Seq data shows intensity or count dependence, respectively (Fig. 29.2). Therefore, a constant fold change criterion is inappropriate as it can produce many false positives at low intensity or

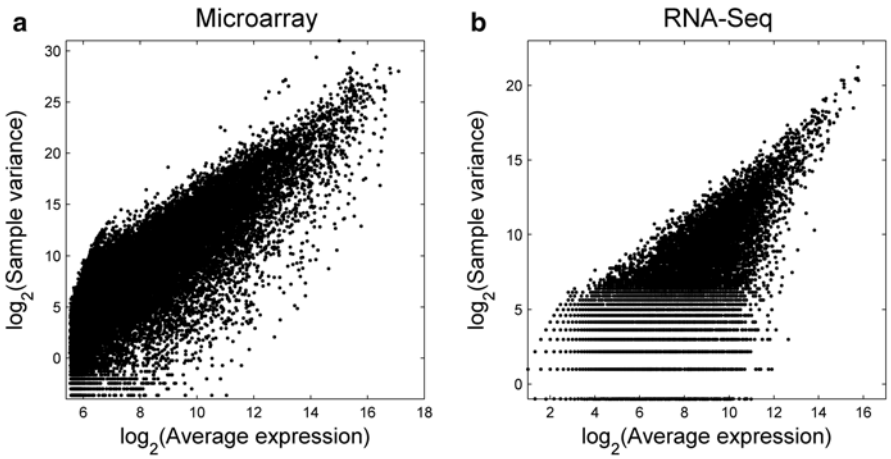

Fig. 29.2 Intensity and count dependent variance structure of (**a**) Microarray and (**b**) RNA-Seq data. Data were taken from (Dennis et al. 2010) for microarray and (Bhargava et al. 2013) for RNA-Seq

count and becomes excessively stringent at high intensity or count. A simple *t*-test can be used to identify differentially regulated genes. However, the *t*-test requires a sufficient number of replicates to estimate the variance reliably. Due to the high cost of experiments, transcriptomic analyses frequently examine low numbers of replicates limiting the use of a simple *t*-test. To address this, modified *t*-tests based on variance-modeling approaches have been proposed to handle small numbers of experimental replicates (Subramaniam and Hsiao 2012; Baldi and Long 2001). In these approaches, the variances are calculated from global or local models of the intrinsic variance structure of the data. These models are based on the assumption that genes with similar intensity or count have similar variance and can be describe as $\sigma^2 = f(\mu)$, where σ^2 represents the variance and μ represents the intensity or count.

Variance-modeling approaches include Variance Modeling with Prior Exponentials (VAMPIRE) (Hsiao et al. 2004, 2005), CyberT (Baldi and Long 2001), and Linear Models for MicroArray data (LIMMA) (Smyth 2004) for microarray data, and DESeq (Anders and Huber 2010) and EdgeR (Robinson et al. 2010) for RNA-Seq data. VAMPIRE and CyberT employ Bayesian estimation of modeled global and local variance, respectively. LIMMA uses linear models for the analysis of differentially expressed genes. In all the above methods developed for microarray, it is assumed that the noise has a normal distribution. In contrast, noise follows a multinomial distribution for count data in RNA-Seq measurements. To account such a noise distribution, EdgeR uses the Poisson distribution and DESeq uses the negative binomial distribution for approximation. VAMPIRE and CyberT are web applications while LIMMA, DESeq, and EdgeR, as well as CyberT, are available as R packages (http://www.r-project.org/).

29.2.2 Protein-DNA Interactomics

Chromatin immunoprecipitation (ChIP) followed by microarray hybridization or sequencing is the most widely used technique to identify genome-wide protein–DNA interactions such as transcription factor (TF) binding sites and histone modifications (Ho et al. 2011). Briefly, after protein-DNA cross-linking, DNA fragmentation and immunoprecipitation with an antibody specific to the target protein, protein bound DNA fragments are isolated and reverse cross-linked. The purified DNA fragments are quantified either through hybridization to (promoter or genome) tiling microarrays (ChIP-chip), or HTP sequencing (ChIP-Seq). When the antibody used is specific to a TF, the analysis provides the information about TF binding sites (TFBS). When the antibody targets histone protein modifications, the information obtained relates to transcriptional activity. Mapping of the intensity of the probes or sequence reads against the whole genome identifies "peaks" in the regions of protein–DNA interaction. The identification of peaks against the background, referred to as peak calling, provides the location of enriched probe intensities or sequence reads in the genome. Peak calling programs include MAT (Johnson et al. 2006) for ChIP-chip, and MACS (Zhang et al. 2008) and PeakSeq

(Rozowsky et al. 2009) for ChIP-Seq data. CisGenome (Ji et al. 2008) is used for both ChIP-chip and ChIP-Seq. A peak list stored during peak calling is further used to annotate the nearest gene(s) using ChIPpeakAnno (Zhu et al. 2010), CEAS (Shin et al. 2009), or Sole-Search (Blahnik et al. 2010). Further, the discovered TFBS or histone binding motifs are represented by position specific weight matrices (JASPAR; TRANSFAC; Reddy et al. 2006) or visualized as sequence logos (Schneider and Stephens 1990; Crooks et al. 2004).

29.2.3 Proteomics

In the past two decades, proteomics has made substantial progress moving from gel based protein analysis to HTP mass spectrometry (MS)-based qualitative and quantitative analysis of the cellular proteome (Tyers and Mann 2003). Various MS based methods have also been developed to study protein composition of organelles, post-translational modifications of signaling proteins, and composition of protein complexes (Walther and Mann 2010; Andreyev et al. 2010a). In all these experiments, protein samples are enzymatically cleaved into short peptides and eluted from high-performance liquid chromatography (LC) for MS/MS analysis. The spectrum obtained from individual peptide analysis is used either for computational de novo sequencing of the peptide or for matching against protein spectrum databases. De novo sequencing algorithms include PEAKS (Zhang et al. 2012) and Lutefisk (Johnson and Taylor 2002) and the widely used protein databases search engines are PEAKS, Mascot (Perkins et al. 1999), SEQUEST (Eng et al. 1994), and X!Tandem (Craig and Beavis 2004). Similar to bioinformatics analysis of transcriptomic data, global, Lowess or quantile normalization is generally used as a normalization method. Over (or under)-representation of protein is identified using simple *t*-test with the assumption that data are normally distributed. LIMMA has also been utilized for the analysis of proteomic data (Ting et al. 2009).

29.2.4 Metabolomics and Lipidomics

Metabolomics addresses biological events downstream from transcription and translation. With the recent development of sensitive mass spectrometry, a large numbers of metabolites have been analyzed under various experimental conditions. Two complementary approaches for sample preparation may be utilized prior to Liquid Chromatography–Mass Spectrometry (LC–MS) analysis: direct infusion (shotgun metabolomics) and metabolite extraction (Dettmer et al. 2007). Direct injection of the samples is used to reduce metabolite losses during the sample preparation step. One advantage of shotgun metabolomics is that it can identify a large number of metabolites; however high protein content in the sample can hinder the LC performance. To avoid this problem of direct injection, or for targeted

metabolomics, solid-phase extraction or liquid–liquid extraction techniques may be used to reduce the complexity of metabolite mixtures. For volatile metabolites such as alcohols, aldehydes, or ketones where solid-phase or liquid–liquid extraction is not feasible, solvent-free techniques with gas chromatography (GC) are used. The bioinformatics analysis includes normalization, peak identification using databases of known or computationally derived metabolites, statistical analysis to quantify significant changes (abundance) between different samples (Shulaev 2006) and, when time-series data are provided, fluxomics modeling of pathways (Gupta et al. 2009).

Metabolomic studies typically target specific classes of metabolites because of the diverse chemical nature of metabolic compounds. Advantages of targeted metabolomics include dependable results for otherwise poorly resolved metabolites (e.g., low abundance) and detection of metabolites lost in shotgun metabolomics. As an example, the area within the field of metabolomics focusing on the study of lipid compounds is known as lipidomics. There are eight major categories of lipid: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, cholesteryl esters, cardiolipins, and prenols (Fahy et al. 2005). All lipids except eicosanoids are extracted from the cell. Eicosanoids are produced and quickly transported to the extracellular space and thus are measured in the media or serum/plasma. All of these categories of lipids require different extraction and measurement protocols. For example, analysis of eicosanoids requires solid-phase extraction from media followed by LC–MS, whereas analysis of fatty acid requires addition of methanol and hydrogen chloride followed by extraction with iso-octane and GC-MS analysis. Further details for the analysis of other category of lipids can be found at the LIPID MAPS consortium website (http://www.lipidmaps.org). For analysis of the spectra obtained, several metabolomics and lipid specific MS analysis tools such as MZmine (Katajamaa et al. 2006), Lipid Data Analysis (LDA) (Hartler et al. 2011), Lipid Mass Spectrum Analysis (LIMSA) (Haimi et al. 2006), Fatty Acid Analysis Tool (FAAT) (Leavell and Leary 2006), and LipidXplorer (Herzog et al. 2011) are available.

29.3 Functional Analysis of Omics Data

29.3.1 Enrichment Analysis

Omics data are biologically interpreted using literature knowledge and the curated annotations available through various databases (Table 29.1). Most commonly, enrichment analysis is used to identify the significantly affected Gene Ontology (GO) (Ashburner et al. 2000) terms and *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (Kanehisa and Goto 2000) or other pathways. For enrichment analysis, two complementary approaches are employed. The first approach calculates over-represented GO terms and pathways among the list of significant features identified from the omics experiments. Many tools such as AmiGO (The Gene Ontology

Table 29.1 Examples of databases for storage and analysis of omics data

Database	Species	Access/web link
Metabolomics or lipidomics		
LIPID MAPS[a]	Mouse	http://www.lipidmaps.org/
Metabolomics workbench	Multi-organism	http://www.metabolomicsworkbench.org/
HMDB	Human	http://www.hmdb.ca/
Transcriptomics		
GEO	Multi-organism	http://www.ncbi.nlm.nih.gov/geo/
Array express	Multi-organism	http://www.ebi.ac.uk/arrayexpress/
LIPID MAPS[a]	Mouse	http://www.lipidmaps.org/
MacGate[a]	Mouse, human	http://www.macgate.qfab.org/index.htm
Proteomics		
GPMDB	Multi-organism	http://gpmdb.thegpm.org/
ProteomeXchange	Multi-organism	http://www.proteomexchange.org/
Peptide atlas	Multi-organism	http://www.peptideatlas.org/
PRIDE	Multi-organism	http://www.ebi.ac.uk/pride/
Protein–protein interactions		
HPRD	Human	http://www.hprd.org/
SGMP	Mouse, human	http://www.signaling-gateway.org
MIPS	Multi-organism	http://mips.helmholtz-muenchen.de/proj/ppi/
STRING	Multi-organism	http://string-db.org/
BIOGRID	Multi-organism	http://thebiogrid.org/
TF or miRNA binding sites		
TRANSFAC	Multi-organism	http://www.gene-regulation.com/pub/databases.html
JASPAR	Multi-organism	http://jaspar.genereg.net/
miRBase	Multi-organism	http://www.mirbase.org/
miRTarBase	Multi-organism	http://mirtarbase.mbc.nctu.edu.tw/
Pathways or ontologies		
KEGG	Multi-organism	http://www.genome.jp/kegg/
Biocarta	Human	http://www.biocarta.com/genes/index.asp
Reactome	Multi-organism	http://www.reactome.org
Gene ontology	Non-specific	http://www.geneontology.org/
MSigDB	Multi-organism	http://www.broadinstitute.org/gsea/msigdb/index.jsp

[a]Macrophage specific databases

database), GOby (part of VAMPIRE suit) (Hsiao et al. 2005), Ingenuity pathway analysis (IPA™) [http://www.ingenuity.com/], and Database for Annotation, Visualization and Integrated Discovery (DAVID) (da Huang 2009a, b) are available as web applications. These database-driven applications store annotation data from several sources, including, GO, KEGG, TRANSFAC (TRANSFAC), and Biocarta (BIOCARTA 2013). Most of these applications use the hypergeometric distribution or Fisher exact test to compute the enrichment likelihoods. The second approach identifies the enrichment of related GO terms and pathway genes (known as gene set) in one end of the ranked data (Subramanian et al. 2005). The data can be ranked

using metrics such as *t*-test, correlation, fold change etc. This approach calculates an enrichment score similar to the Kolmogorov–Smirnov statistic based on the ranking of the genes from the gene set and estimates the statistical significance by using a random permutation. As an example, GOby was used in the analysis of gene expression data in RAW264.7 macrophages treated with Kdo2-Lipid A (KLA) and/or Compactin (Dennis et al. 2010). Results showed the enrichment of Toll-like Receptor (TLR), Jak-Stat, NF-κB, and cytokine–cytokine receptor interaction KEGG pathways.

29.3.2 Motif Analysis

A motif is a short DNA (or RNA) sequence to which TF (in case of DNA) or miR (in case of mRNA) bind, thus enabling it to influence the expression of its target gene. Databases such as JASPAR and TRANSFAC report known TFBS as well as Position Weight Matrices (PWMs) for TFs, which describe their DNA-binding profiles. These matrices can be used to scan gene promoter regions to identify new TFBS using two complementary approaches. The first approach finds the alignments using PWMs on the promoter sequence and assigns statistical significance compared to random sequences based on nucleotide composition of the genome (Bailey 1994). The second approach finds enrichment of an aligned motif in the target promoter set compared with the background set and matches the enriched motifs to known TFs motifs to determine the corresponding TFs (Halperin et al. 2009). Commonly used tools include MEME (Bailey 1994), Allegro (Halperin et al. 2009), and Weeder (Pavesi et al. 2004). Details of other software tools and their comparison can be found in (Tompa et al. 2005). In contrast to TF which bind DNA and regulate transcription, miR regulates protein expression through base-paring with its target mRNA (Bartel 2004). miR motif prediction tools follow miR-target interactions rules such as sequence matching, thermodynamics of miR:RNA binding etc. and include TargetScan (Grimson et al. 2007), miRanda (Betel et al. 2008; Enright et al. 2003) PicTar (Krek et al. 2005), and DIANA MicroT Analyzer (Kiriakidou et al. 2004).

29.3.3 ANOVA

Datasets from large-scale multi-factor (time, ligands, etc.) experiments can be analyzed using the analysis of variance (ANOVA) approach. In ANOVA, the observed variance in the measured data is partitioned into the effect of individual factors or treatments (Churchill 2004). A *p*-value is assigned to the effect of individual factors included in the model. ANOVA has been used to factor out the significance of different treatments or time-effects on experimental measurements of genes (Draghici et al. 2003), proteins (Pradervand et al. 2006), and metabolites (Dennis et al. 2010).

ANOVA results may be further analyzed by performing multivariate analyses such as principal component analysis (PCA) on the interaction terms for different factors (de Haan et al. 2007) to determine if such interactions may be significant under certain conditions. Bi-plots from PCA may also aid in visualization and interpretation of results. More recently, the combined approach of ANOVA-PCA has gained considerable attention from statisticians, especially when three or more factors need to be analyzed (Climaco-Pinto et al. 2009).

29.3.4 Correlation and Clustering Analysis

29.3.4.1 Pearson Correlation

Pearson correlation is widely used to find which variables show similar changes across different experiments or time-points (Egghe and Leydesdorff 2009; Anderson 1984). Pearson correlation coefficients can also be used to perform hierarchical clustering (Langfelder et al. 2008) and to generate correlation networks (Langfelder et al. 2008; Fukushima et al. 2011; Johansson et al. 2011; Adourian et al. 2008). Such networks may capture some aspects of the causality among variables or factors. A more elaborate discussion on the issue of correlation versus causality is presented elsewhere (Maurya and Subramaniam 2009).

Correlation analysis has been applied to various macrophage systems to elucidate how different molecular components function in a network and to understand their phenotypic similarities and differences. For example, Tsuchiya et al. (2009) have used genome-level correlation analysis on gene data from four different genotypes of macrophages stimulated with LPS to gauge the similarities between them. Ramsey et al. (2008) have used time-lagged correlation analysis on gene expression data from macrophages stimulated with LPS and other ligands to develop coexpression networks. Further, they performed an integrated analysis of gene expression data with TF-target gene information and identified context-specific enriched regulatory motifs and novel causal relationships between TFs and target genes.

Correlation analysis has also been used in several clinical studies to find putative links between specific molecular data and higher level phenotypes and diseases (McNearney et al. 2004; Ludwig et al. 2000; Cappuzzello et al. 2011; Takahashi et al. 2010). Feihn and coworkers (Kose et al. 2001; Roessner et al. 2001) have used metabolite–metabolite correlation analysis-based clustering and PCA to develop and visualize data-derived metabolic networks.

29.3.4.2 Time-Interval Weighted Correlation

In many biological experiments, the measurements are taken at non-uniform time-intervals (more frequently at the beginning and less frequently at later time-points). In these cases, a weighted correlation in which the time-points are weighted proportional to the time-interval is more appropriate than the raw correlation described

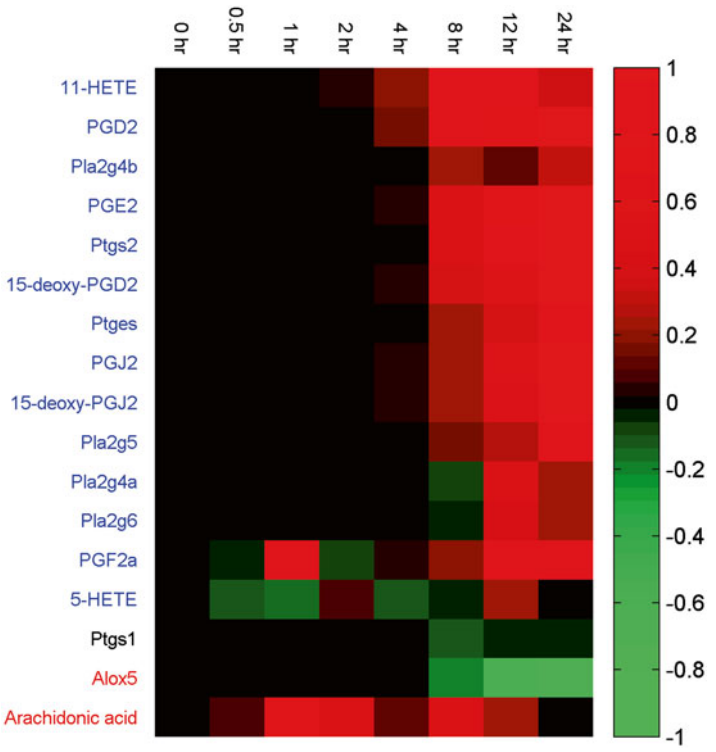

Fig. 29.3 Heat map for data from eicosanoids (measured from media) and eicosanoid biosynthesis related genes indicating good correlation between the time courses of lipids and genes (with permission). The data are fold change of KLA treatment vs. control. Each row is normalized. The names of the lipids and genes displayed on the *y*-axis are listed in *different colors* to indicate the clusters

above. Such an approach has been used recently to identify interesting relationships between transcriptomic and lipidomics changes in RAW 264.7 macrophages upon treatment with KLA (Dennis et al. 2010). They have reported that the temporal changes in prostaglandin (PG) lipids (e.g., PGE_2, PGJ_2, and $PGF_2\alpha$) and the prostaglandin synthase genes (Ptgs2 and Ptges) were similar, as reflected in the good correlation among them (Fig. 29.3). Similar observations were made for the temporal changes in the levels of various sterol lipids and the corresponding genes. These results indicate that joint-correlation analysis can potentially uncover such underlying cellular mechanisms (Dennis et al. 2010).

29.3.4.3 Clustering

Many clustering techniques have been described, all of which assign elements to a cluster based on a defined similarity measure comparing all pairs of elements. Hierarchical clustering increasingly merges clusters of sufficiently high similarity

(agglomerative), or iteratively splits clusters to disconnect vertices with low similarity (divisive). K-means clustering, an example of partitional clustering, requires prior specification of k, and separates nodes into k clusters that minimize the total intra-cluster distance (dissimilarity) as measured relative to each cluster's center of mass (centroid). Spectral clustering employs k-means clustering after transforming the input data using the eigenvectors of the similarity (or other) matrix. Fuzzy clustering variants allow for inclusion of nodes in more than one cluster. Various other approaches to clustering have been reviewed (Gan et al. 2007; Jain et al. 1999).

A software suite for analyzing gene expression data, WGCNA (Weighted Gene Correlation Network Analysis), was developed by Langfelder and Horvath (2008). Given a data matrix comprised of multiple variables such as genes or proteins, the method computes a correlation matrix and converts it into an adjacency matrix representing a network of significance correlations exceeding a specified threshold. The correlation coefficients are used as the edge-weights. The software includes functions to build the expression correlation network and cluster the genes using hierarchical clustering; a branch-cutting method (to detect modules) and functions to find biologically significant modules, describe topological properties and to visualize results are also included. Rau et al. (2013) have developed a method for network analysis called maximal information component analysis which uses mutual information besides the correlation measure and they have compared their approach with WGCNA on macrophage and liver specific data. WGCNA has also been applied to gene expression data sets from disease conditions such as epilepsy (Winden et al. 2011), diabetic kidney disease (Tang et al. 2012), and HIV-associated cognitive impairment (Levine et al. 2013), among others.

29.3.5 Data-Driven Network Reconstruction

Inferring the topology of functional networks such as gene regulatory and signaling networks from large-scale experimental data is necessary for extracting higher level and mechanistic knowledge (Maurya and Subramaniam 2009). Accordingly, several researchers have embarked on the development of methods for data-driven network reconstruction and their further integration and interpretation with legacy knowledge (Asadi et al. 2012). Popular techniques for data-driven network reconstruction include regression and dimensionality-reduction based methods (e.g., statistical significance tests combined with either principal components regressions (PCR), or partial least-squares (PLS) (Gupta et al. 2010; Pradervand et al. 2006), partial-correlation-related analyses (Schafer and Strimmer 2005), Bayesian networks (Janes et al. 2005; Sachs et al. 2005), and hybrid methods such as Linear Matrix Inequalities (LMI) (Montefusco et al. 2010) and Least Absolute Shrinkage and Selection Operator (LASSO) (Tibshirani 1996; Bonneau et al. 2006). Matrix-based approaches (Famili et al. 2005; Karnaukhov et al. 2007) have also been proposed. A detailed review of various network-reconstruction methods can be found in Maurya and

Subramaniam (2009). Several case studies have applied network-reconstruction approaches to study macrophages and other cells of the immune system and their related functions. For example, a PLS approach has been used to identify the interaction of apoptotic and pro-survival signals in cellular apoptosis (Janes et al. 2005). An alternative algorithm proposed by Pradervand et al. (2006) enables one to perform an input–output mapping by utilizing steady-state or time-averaged data. The algorithm was used to identify lumped networks representing regulation of cytokine production and release by signaling pathways using phosphoprotein signaling (inputs) and cytokine (output) measurements in RAW 264.7 macrophage cells stimulated with various single and double-ligand combinations in experiments performed by the Alliance for Cellular Signaling (AfCS) (The Alliance for Cellular Signaling; Dinasarapu et al. 2011). Use of temporal data to reconstruct biological networks in macrophages has been illustrated by Bonneau et al. (2006) and Gupta et al. (2010).

29.3.6 Analysis of Biological Networks

HTP techniques to identify protein–protein interactions have provided large interaction data sets for both human (Ewing et al. 2007; Rual et al. 2005; Stelzl et al. 2005) and model eukaryotic species (Giot et al. 2003; Ito et al. 2001; Li et al. 2004; Uetz et al. 2000). The main HTP techniques used are yeast-two-hybrid (Y2H) (Fields and Sternglanz 1994) and tandem affinity purification combined with mass spectrometry (TAP-MS) (Gavin et al. 2002). Using experimental data, particularly for species less comprehensively studied (Suzuki et al. 2001), protein–protein interactions have been extended by computational refinement of predictions based on evolutionarily conserved interactions of identified orthologs, gene coexpression patterns, known domain–domain interactions, genomic context, and literature mining (Yellaboina et al. 2008; Li et al. 2010; von Mering et al. 2005). In addition to protein–protein interactions, experimental and predicted interactions between transcription factors or miRs and their target genes are being assembled, and there is an abundance of biochemical data documenting enzyme-substrate interactions, all of which can be incorporated into interaction networks. Table 29.1 provides a list of several interaction databases, most of which are publicly available.

The identification of network modules or clusters provides a useful method for extracting biologically important information from such large, otherwise intractable, networks. Modules may be defined as regions within the network that are densely connected, and/or whose member nodes are more similar to each other, by a chosen similarity measure, than they are to nodes external to the module. Depending on what nodes and edges represent, such clusters may represent, for example, groups of proteins that physically interact to form a functional complex or pathway, or that share common structural motifs; or groups of genes whose correlated expression may suggest coordinated regulation. Integration of additional data

sources beyond that encoded in the static network may enable identification of protein complexes whose components are overexpressed in a particular condition, or which harbor multiple gene mutations identified in a particular disease such as cancer (Lin et al. 2007). The topology of such modules itself may provide important information, as nodes (genes, proteins, or metabolites) which are highly connected to other module members may play a central role in the module's function. Taking a larger view of how individual clusters overlap can provide insight into the modular construction of pathways and how this may change under different conditions.

There are two fundamental approaches to identify subgraphs of interest: partitioning methods and module-finding methods. Partitioning methods assign every node to one and only one partition, or community, though some partitions may contain only a single node. In contrast, module-finding methods do not necessarily assign every node to a significant cluster, and conversely, some nodes may be included in more than one cluster.

Graph partitioning is a well-studied problem and numerous partitioning algorithms exist. This group includes clustering techniques which require definition and calculation of a similarity measure between all pairs of nodes. These techniques, some of which have been described above (Correlation and Clustering Analysis), may be applied to networks whether or not they are sparse (numbers of edges and nodes are of the same order). Other methods of graph partitioning, which identify structural communities, require the network to be sparse. These approaches include divisive algorithms, such as the well-known edge betweenness algorithm of Girvan and Newman (Girvan and Newman 2002; Newman and Girvan 2004), and modularity optimization techniques (e.g, greedy optimization, simulated annealing, and spectral optimization). A novel force driven approach analogous to modularity finding has been introduced recently (Narayanan and Subramaniam 2014). A recent review Fortunato (2010) comprehensively discusses the various approaches to network partitioning, and discusses open problems as well as how to test algorithms and evaluate the significance of partitions.

Depending on the method of construction, biological networks may have a significantly greater number of edges than the number of nodes, and may thus be less amenable to biologically meaningful partitioning into non-overlapping communities. Accordingly, module-finding algorithms have been developed aimed at identifying clusters of nodes that share significant function, regulation, or structure. The first module-finding algorithm applied to large protein interaction networks, MCODE, identifies densely connected regions by weighting vertices according to their local network density, then selecting the highest unseen weighted vertex and recursively adding connected vertices that meet a pre-defined weight threshold (Bader and Hogue 2003). Figure 29.4 illustrates a densely connected subnetwork identified by MCODE in a large mouse protein–protein interaction network.

Methods have also been developed to integrate protein interaction networks and gene expression data to detect subnetworks that display correlated expression, suggesting shared functionality, or that distinguish between different perturbations or disease states that may point to underlying mechanisms or a basis for classification.

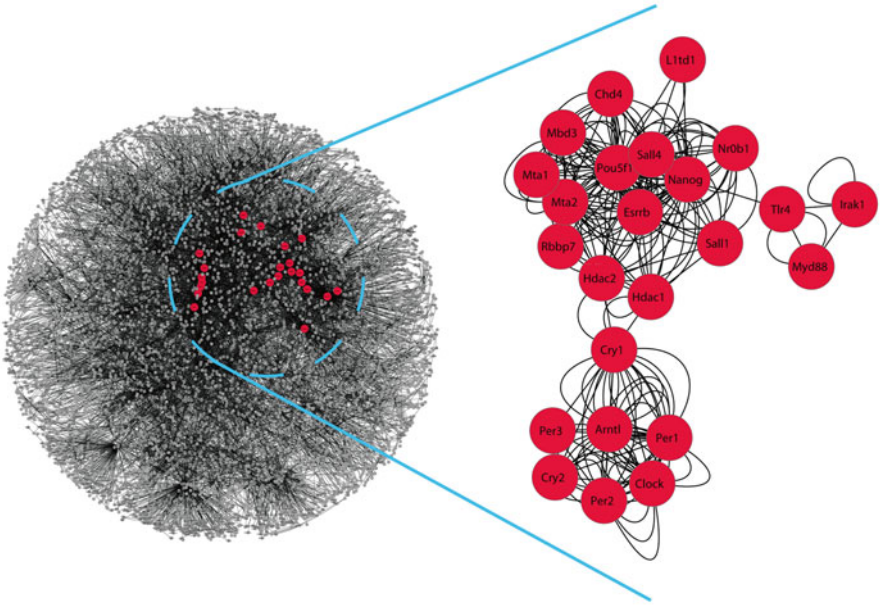

Fig. 29.4 Cluster identification using MCODE in Cytoscape. The BIOGRID mouse interaction network (5,534 nodes, 12,919 edges) was downloaded using Cytoscape 3.0.0 and rendered using the Spring Embedded algorithm. A single large connected component was identified and extracted (5,153 nodes, 12,580 edges) and submitted to the MCODE app for identification of densely connected subgraphs. The figure shows the connected component with nodes included in the highest-scoring MCODE cluster (24 nodes, 235 edges) enlarged and colored *red*. The entire component has a mean of 2.44 edges/node and the MCODE cluster a mean of 9.79 edges/node. Functional enrichment analysis using DAVID indicates that 23 of the 24 nodes are annotated for "regulation of transcription" (GO Biological Process)

Many of these may be extended to integrate other types of large-scale data, when available. One of the earliest methods, jActiveModules (Ideker et al. 2002) converts each gene's *p*-value of expression change to a z-score, calculates a subnetwork's aggregate z-score, and uses a simulated annealing-based search strategy to find subnetworks with the best statistically significant (relative to same-sized random gene sets) aggregate scores. These (transcriptionally) "active" modules are expected to be of strong biological interest. PinnacleZ (Chuang et al. 2007), developed for cancer classification, greedily searches for subnetworks whose aggregated expression-based activity score significantly distinguishes between the two phenotypes under study. MCODE, JActiveModules and PinnacleZ, as well as many other cluster-finding and integrative algorithms have been implemented as apps (plug-ins) for Cytoscape (Cline et al. 2007; Smoot et al. 2011), an open source platform for complex network assembly, analysis, visualization, and (biological) annotation. The Matisse software suite includes algorithms for identifying functional modules using protein (or other)

interaction networks and gene expression data. The basic Matisse algorithm Ulitsky and Shamir (2007) identifies connected subnetworks that contain member nodes with highly correlated gene expression patterns; it comprises of three steps: detection of small, high scoring gene set seeds, seed optimization, and significance-based filtering. The Matisse heuristic has been modified by redefining the similarity score to identify subnetworks whose member nodes have highly correlated gene expression patterns as well as high correlation with a clinical (phenotypic) parameter (Ulitsky and Shamir 2008), and by adjusting seed identification and optimization to identify detect subnetworks with correlated expression patterns whose member nodes are connected with high-confidence interactions (Ulitsky and Shamir 2009). Also included is Degas (Ulitsky et al. 2010), which detects subnetworks that contain multiple (transcriptionally) dysregulated genes across a set of disease vs. control samples. Whereas all the forgoing integrative algorithms incorporate heuristics, Dittrich et al. (2008) describe an exact solution to identifying differentially expressed subnetworks based on integer-linear programming (ILP).

29.3.7 Dynamic Modeling

Cellular processes are inherently dynamic. Dynamic modeling is an important area in systems biology to study the role of various proteins and metabolites in cellular function and their relationship to health and disease. In the past 15 years, several software packages have been developed to facilitate dynamic modeling. An extensive list of such software is available (Hucka et al. 2003; The Systems Biology Markup Language) and include CellML (Miller et al. 2010), JSim (a java-based simulation and animation environment) (Miller et al. 1997), VCell (Schaff et al. 1997; The Virtual Cell), Systems Biology Workbench (Sauro et al. 2003), COPASI (Hoops et al. 2006), and MCell (Casanova et al. 2004). These tools allow kinetic modeling and time-course simulation, steady-state analysis, sensitivity analysis, and parameter estimation. Some of these also can perform stochastic simulation and account for spatial variation (partial differential-equation-based modeling). Many of these software packages provide Systems Biology Markup Language (SBML) format-based model exchange. In a recent review (Subramaniam et al. 2011), we have summarized the salient features of these modeling tools, which may help the modeler choose the most appropriate software for a given problem.

Dynamic modeling has been applied to several metabolic and signaling pathways. Examples of metabolic pathways include genome-scale metabolic networks for different organisms such as *Saccharomyces cerevisiae* (the budding yeast), *Escherichia coli*, and human (Forster et al. 2003; Feist et al. 2007; Duarte et al. 2007; Burgard et al. 2004; Song et al. 2013; Young et al. 2008). Callender et al. (2010) have developed a model of diacylglycerol dynamics in the RAW264.7 macrophage. Yang et al. (2007) have developed a model of arachidonic acid metabolism in human polymorphonuclear leukocytes. All of these models generally suffer from

the unavailability of sufficiently large datasets. Gupta et al. (2009) have developed models of macrophage lipid metabolism using time-course experimental data made available by the LIPID MAPS consortium. The rate parameters were estimated using experimental data through a matrix-based approach and optimization. Using these dynamic models, the fluxes in different branches of the network and enzyme activity/rate constants could be computed (Gupta et al. 2009). Examples of signaling pathways include modeling of Mitogen Activated Protein (MAP) kinase pathway in NIH 3T3 cells (Bhalla et al. 2002), regulation of budding yeast cell-cycle (Chen et al. 2000), and calcium signaling in RAW 264.7 macrophages (Maurya and Subramaniam 2007).

29.3.8 Visualization

Given the large datasets generated by omics experiments, a critical element in facilitating understanding and communication of analysis outcomes is the ability to clearly and intelligently visualize the results. Several visualization software applications are publicly available that enable the user to import or construct biological pathways and networks, to project annotation or experimental data upon the pathway/network nodes, and to modify the appearance of the final pathway/network. Examples of visualization software include VANTED (Junker et al. 2006), Pathway Editor (The Systems Biology Markup Language; Byrnes et al. 2009), and Cytoscape (Smoot et al. 2011). Both Pathway Editor and VANTED have Java-based GUIs providing a comprehensive range of viewing and import/export formats. The Pathway Editor has a particularly useful feature that enables the association of multiple time-point data with a single node. In addition to enabling construction, visualization and data projection for networks, as well as pathways, Cytoscape, through its many user-contributed apps, provides tools for modeling and analyzing molecular networks, including calculation of network properties, module identification, and functional enrichment analysis (Cline et al. 2007). Interactions can be imported and exported using a variety of file formats from either user data or interaction repositories. Nodes and edges can be added manually and can be associated with metadata useful for visually distinguishing subsets of interest. A wide range of visual attributes for nodes and edges is available, as are numerous automatic graph layouts that highlight different features of a network. Examples of networks generated using these tools are presented in Fig. 29.5.

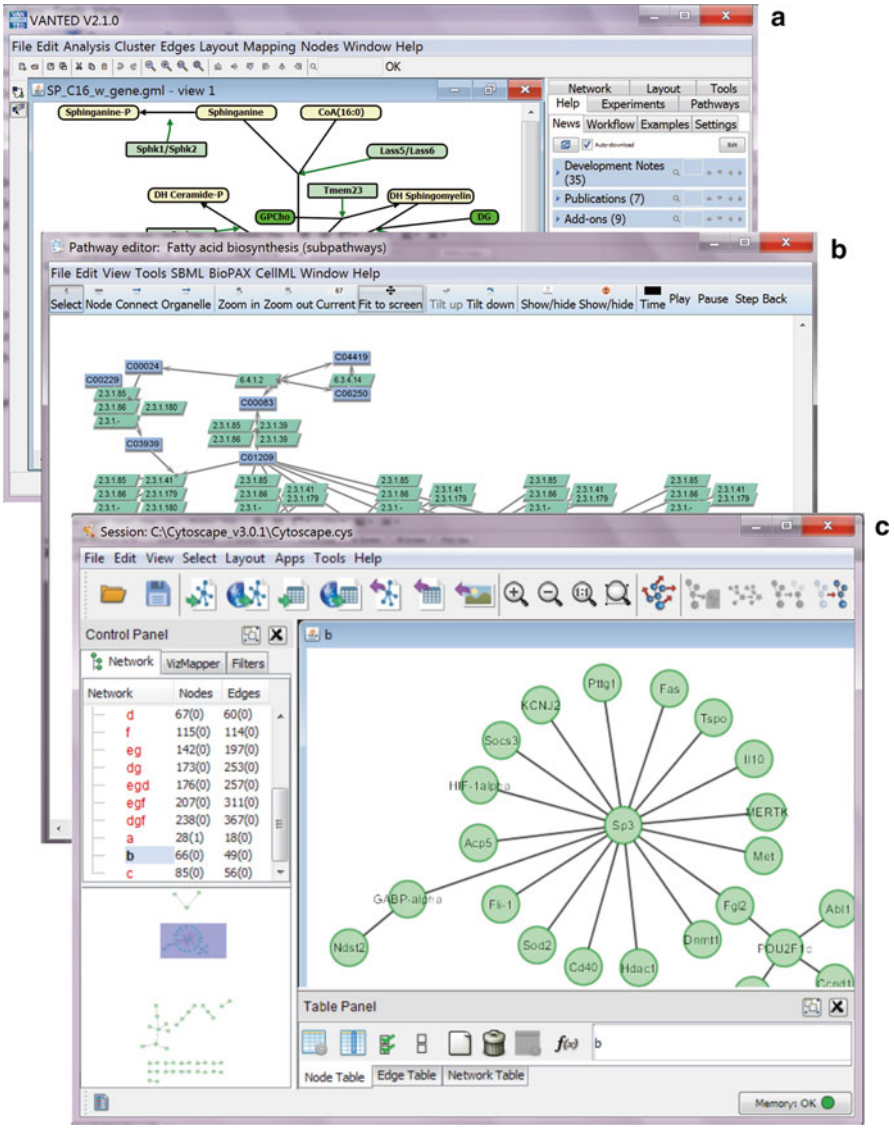

Fig. 29.5 Screen shots of biological network drawing and visualization tools: (**a**) VANTED, (**b**) Pathway editor, and (**c**) Cytoscape

29.4 Macrophages Omics in Biology and Disease

Macrophages are well known to play important roles in immunity and disease as well as embryonic development, homeostasis, and wound repair (Pollard 2009). Omics analyses of macrophages have been useful in augmenting the mechanistic

insights provided by earlier small scale studies. A mouse macrophage cellular and subcellular organelle Lipidome was identified using HTP-MS (Dennis et al. 2010; Andreyev et al. 2010b). Lipids from various categories such as eicosanoids, sterols, and sphingolipids were quantitated and an integrated network was developed showing their interconversion (Dennis et al. 2010) (Fig. 29.6). Using this dataset, flux-based dynamic models of eicosanoid metabolism (Gupta et al. 2009) and sphingolipid metabolism (Gupta et al. 2011) were developed. Parametric sensitivity analysis and validation of some of the estimated rate parameters was also performed. Parametric sensitivity analysis suggested that the response (lipidomic changes) of the system was robust to such perturbations. In a similar study of macrophages by other researchers, parallel gene expression and ChIP-seq analyses identified a list of immediate/early (I/E)- and late-response genes in primary macrophages treated with KLA (Escoubet-Lozach et al. 2011). An integrated transcriptomic and lipidomic analyses showed the role of accumulated desmosterol in suppression of inflammatory-response in macrophage foam cells (Spann et al. 2012). A recent study, using ChIP-seq and lipidomic analysis showed that macrophages stimulated with IFNγ (or CMV infection) promotes direct binding of Stat1 on the Ch25h gene promoter and increases the synthesis and secretion of 25-hydroxycholesterol (Blanc et al. 2013). Combined transcriptomic and lipidomic analyses revealed synergistic effects between KLA-priming followed by ATP-stimulation of bone-marrow derived macrophages (BMDM), mediated by NF-kB and AP-1 acting via their targeted cytokines, to activate both STAT1 (antimicrobial M1 phenotype) and STAT3 (antiproliferative, apoptotic M2 phenotype) which also upregulate glycerolipid and eicosanoid metabolism, respectively (Dinasarapu et al. 2013). In other work, a genome-scale macrophage metabolic model was constructed and simulations were performed which predicted a higher glycolytic capacity in M1 macrophages and greater dependence on oxidative phosphorylation in M2 macrophages, as well as different mechanisms for inhibition of macrophage activation by tryptophan uptake and nucleotide production. Transcriptomic, proteomic, and metabolomic analysis of LPS-stimulated RAW 264.7 macrophages confirmed down-regulation of metabolic genes, proteins, and metabolites associated with metabolic suppression by nucleotide metabolism and tryptophan catabolism (Bordbar et al. 2012).

Cancer is an example of a disease in which macrophage activity may have an important effect on outcome. In most cancers, tumor-associated macrophages (TAM) promote tumor growth and metastasis, though in some cases, most notably colon cancer, macrophages are associated with a survival advantage (Heusinkveld and van der Burg 2011). Microarray studies have helped elucidate many of the mechanisms through which TAM's affect tumor biology. DcR3 (Decoy Receptor 3), upregulated in many tumors, mediates down-regulation of TAM major histocompatibility complex (MHC)-II, required for antigen presentation, via ERK- and JNK-induced deacetylation of CIITA (MHCII transactivator) promoters (Chang et al. 2008). A LYVE-1+ (lymphatic endothelium-specific hyaluronan receptor) subset of M2-TAM is induced by IL-4, glucocorticoids, and tumor-conditioned media, and expresses MS4A8A whose induction via p38/MAPK signaling enhances tumor

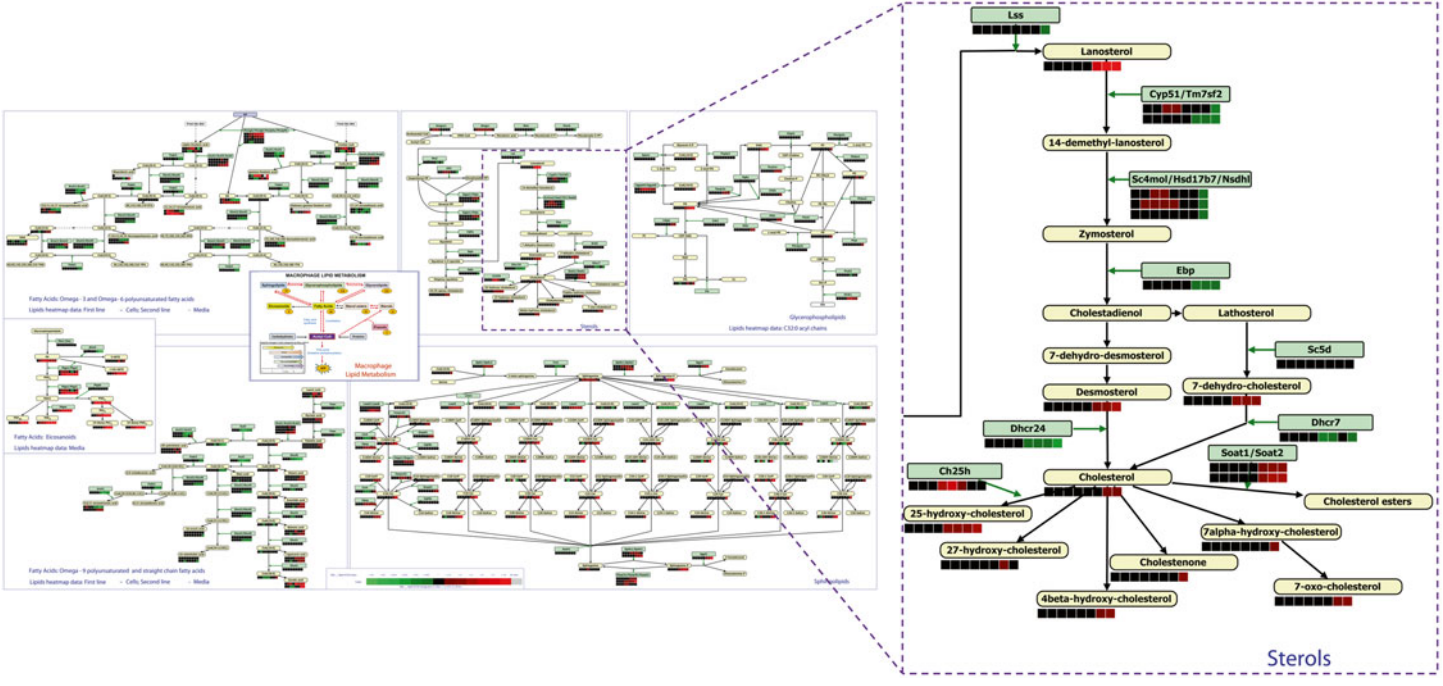

Fig. 29.6 Integrated pathway diagram of lipid metabolism showing representative time courses of lipid metabolite and gene changes in macrophages treated with KLA (Dennis et al. 2010) (with permission). *Inset in the right panel*: a part of the sterol pathway showing biosynthesis and conversion of cholesterol

growth rate (Schmieder et al. 2011) Ets2 deletion in breast-cancer TAMs decreased the frequency and size of lung metastases related to reduced angiogenesis occurring due to removal of Ets2 repression of several known inhibitors of angiogenesis (Zabuawala et al. 2010). Compared with non-specific TAMs, invasion-promoting TAMs express genes related to tissue and organ development, and are enriched for Wnt-signaling molecules that may help mediate a TAM-dependent link between angiogenesis and tumor invasion (Ojalvo et al. 2010). Compared with Myeloid-derived suppressor cells (MDSC) from peripheral blood, monocytic tumor-infiltrating MDSC produced increased levels of CCR5 ligand chemokines, which attract CCR5-bearing Tregs that suppress the antitumor immune response (Schlecker et al. 2012). In contrast, expression profiling has shown that human monocytes co-cultured with a colorectal tumor line differentiate into macrophages and foster down-regulation of genes associated with proliferation and up-regulation of genes associated with apoptosis (Ong et al. 2012).

29.5 Perspective and Future Direction

Biological responses of macrophages reflect a coordinated interplay of proteins, genes, and metabolites. Quantification of all relevant types of omics data is only a first step toward the identification of interactions or mechanisms. Not only quantification but also specific localization information is important for mechanistic interpretation. There has been significant progress in developing sophisticated methods to measure omics data at the cellular level. However, methods for obtaining omics information at subcellular and single cell levels are presently lacking. This constitutes the first challenge that future omics experiments must address. Furthermore, the quality of biological interpretation of omics data depends upon literature knowledge and curated databases. Often, these databases are curated at the level of the organism, thus missing context-specific information residing at the tissue or cellular level. These databases are also deficient in curating the subcellular localization and cross-interaction of proteins, genes, and metabolites. Thus, the second challenge requires curation of tissue-specific cellular and subcellular level information and integration of gene regulatory, protein signaling, and metabolic pathways for context-specific systems-level analysis. While various sophisticated computational methods have been developed for each type of data, there is a need for compatible methods able to analyze and integrate different kinds of data. Thus, the third challenge lies in development of methods for integrating various types of omics data. Finally, the high cost of omics experiments presently remains a challenge. With decreasing cost and increasing computational power, it is expected that in the coming decades new experimental methods and systems biology approaches will be developed that will go far in identifying mechanistic underpinnings of biological function and their dysregulation in disease.

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