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The Macrophage as Therapeutic Target

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Preface

During the past decade, the rapid growth of molecular and cellular knowledge of macrophages (MØ), as a specialized host defence and homeostatic system, has begun to offer attractive targets for therapeutic intervention. MØ play a central role in a wide range of disease processes, from genetically determined lysosomal storage diseases, to acute sepsis, chronic inflammation and repair, tissue injury and cell death. Under- or overactivity of MØ clearance, immune effector functions and responses to metabolic abnormalities contribute to common disorders such as autoimmunity, atherosclerosis, Alzheimer's disease and major infections including AIDS and tuberculosis.

The discovery of tumour necrosis factor (TNF)-α and the development of specific inhibitors that are highly effective clinically in diseases such as rheumatoid arthritis illustrate the way scientific advances have already been translated into practice. Development of powerful molecular genetic methods made it possible to clone and express specific cytokines produced by MØ and potent growth factors acting on MØ and other myeloid cells, such as granulocyte–macrophage colony-stimulating factor (GM-CSF), to modulate and boost MØ activities. Specific plasma membrane receptors control cell recruitment, adhesion, endocytosis and activation of innate and acquired immune functions. Discovery of the Toll-like receptors has already served as a major spur to uncover signalling pathways that might yield future therapeutic targets. Phagocytic uptake of apoptotic cells and micro-organisms by MØ contribute to host defences, as well as providing a potential niche for intracellular pathogens.

Whilst the goals of therapeutic intervention based on improved understanding of MØ functions and their contribution to pathogenesis may seem self evident, there are considerable difficulties in producing useful new agents. The MØ of the body constitute a distributed cellular system also known as the mononuclear phagocyte, or reticulo-endothelial system, with great heterogeneity in cell differentiation and activation in different organs and disease states. Common progenitors give rise to tissue MØ which differ considerably in their properties, e.g. in the nervous system and liver, to dendritic cells, specialized for antigen presentation to naive T lymphocytes and to osteoclasts, multinucleated cells able to resorb living bone. Their receptors and versatile biosynthetic and secretory responses result in adaptation to very different microenvironments. Their

systemic actions help to integrate many physiologic functions and pathologic processes. It should therefore not come as a surprise if MØ contribute beneficial as well as deleterious roles to the diseased host. They represent the classic two-edged sword. It is a formidable challenge to aim selective intervention at subpopulations of cells or subsets of their gene products without affecting their vital normal functions or other cells, directly or indirectly. A further difficulty is our present-day ignorance of basic mechanisms of MØ functions, e.g. in vaccine development (their role as natural adjuvants or immunosuppressants) and their complex life history in vivo. Gene ablation and transgenesis have confirmed some hypotheses and left others open. Cell culture models are useful, but imperfect, in not mimicking complexity within the host. Classic pharmacologic approaches need to be aligned with newer knowledge of MØ gene expression and regulation.

The present volume covers a range of subjects and provides opportunities for a more focused MØ-targeted approach. The individual chapters review selected topics briefly, to place cellular processes and molecular targets in perspective. These are grouped broadly. Section I deals with general issues of cell differentiation, routes of delivery and of MØ-specific gene targeting, with particular emphasis on the living host. Section II deals with selected plasma membrane receptors, uptake processes, regulation of cellular responses and different categories of secretory products. Finally, Sect. III considers specialized cell types, environments and examples of cell-pathogen interactions. Overall, the volume should provide a broad sample of the state of the art. Useful reviews and references in the literature are cited within individual chapters.

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Oxford, Spring 2003 Siamon Gordon

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(their addresses can be found at the beginning of their respective chapters)

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Part 1
General

The Macrophage as a Validated Pharmaceutical Target

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Abstract "Therapeutic validation" is a utilitarian classification of the application of the basic science base of the macrophage. Reduction to therapeutic practice represents the cutting edge of therapy, but rests upon a decades-old basic science foundation. Macrophage-targeted therapeutics have now added significant value to the lives and quality of life of patients, without undue adverse effects in multiple disease settings. These are exemplified by the impact of macrophage enzyme replacement for a lysosomal storage disease (Gaucher's), the modulation of osteoclast-dependent bone destruction by bisphosphonates, and revolutionary impact of TNF sequestrants on both rheumatoid arthritis, as well as the delineation of new mechanisms in the understanding of Crohn's diseases. The macrophage, as a cell, is now beginning to reach a full measure of therapeutic maturity in the application of the understanding of the particular rate-limiting roles that it plays in the maintenance of health or the induction of diseases.

Keywords Bisphosphonates, Crohn's disease, Enzyme replacement, Gaucher's disease, Macrophage, Osteoporosis, Rheumatoid arthritis, TNF sequestration

The concept of therapeutic target validation bears disproportionate weight in the conduct of applied science in the pharmaceutical and biotechnology sectors, where research decisions are significantly influenced by perceptions of market size. These organizations measure the success of their scientific output by products launched and the expansion of revenues earned and not by the successful testing of meaningful hypotheses and the resulting high-impact publications. To do this, however, the products must add significant value to the lives and quality of life of patients, without undue adverse effects. The ability to regard the macrophage as a "validated" therapeutic target suggests the macrophage, as a cell, is now beginning to reach a full measure of maturity in the application of the understanding of the particular rate-limiting roles that it plays in the maintenance of health or the induction of diseases.

The notion of "therapeutic validation" is simply a utilitarian classification of the application of the basic science base of the macrophage. In many respects, the reduction to therapeutic practice may represent the cutting edge of therapy, but rests upon a decades-old basic science foundation. It provides an objective, although retrospective, validation of the importance of the study of macrophages to advancing human therapeutics. The view is, however, skewed towards the major medical needs that drive economic activity, and does not necessarily reflect the full contribution of the study of macrophages, where the advancing science base has had major impacts upon the understanding of immunity, vaccination, tissue remodeling, and embryology, although in areas that have not yet been brought to therapeutic fruition. In this essay, I have used a broad definition of macrophage study that includes cells of the monocytic lineage such as monocytes, macrophages, osteoclasts, and have also included discussion of inherited metabolic defects in which macrophages are central to the expression of tissue pathology and dysfunction. I will also show how the quality of the basic science base was prospectively predictive of both efficacy and the adverse experience profiles associated with therapeutic approaches to molecules affecting macrophage function.

1
Modification of Macrophage Function Can Now Be Said to Modify Disease Outcome, Rather than Provide Symptomatic Relief

The validation of the macrophage as a proven therapeutic target has crossed multiple treatment modalities. In the macrophage-specialized function of lysosomal degradation, the lysosomal storage defects of Gaucher's disease have led to the relatively successful replacement of a heritable defective enzyme, glucocerebrosidase, using recombinant enzyme, as an orphan disease. In contrast, the treatment of rheumatoid arthritis and Crohn's disease has been revolutionized by the advent of tumor necrosis factor (TNF) sequestrants. These have not only improved patients' lives but have provided unique insights into human disease mechanisms not readily modeled in animal models (Podolsky 2002). The successful treatment of osteoporosis and Paget's disease of bone by the inhibition

of osteoclast-dependent bone resorption represents the successful use of small molecules (bisphosphonates) in the alteration of outcome of an event entirely dependent upon cells of the monocytic lineage. These all represent highly specific macrophage-related events in pathology usefully modulated therapeutically, and are much more compelling evidence for the specific role of macrophages in pathology than is, for example, the expression and upregulation of cyclooxygenase 2 in macrophages, where therapeutic efficacy is only partly attributable to macrophage effects, and where such efficacy is largely symptomatic, rather than disease modifying in terms of long-term impact upon patient outcomes.

2
Enzyme Replacement in Gaucher's Disease

Gaucher's disease is an autosomal recessive disease with its highest prevalence (type I) in Ashkenazi Jews (Balicki and Beutler 2002). It is a typical lysosomal storage disease caused by a deficiency in glucocerebrosidase, an essential step in the cleavage of glucose from ceramide (Inoue and Lupski 2002). Glucocerebroside substrate, therefore, accumulates within cells of the mononuclear phagocyte system and within the central nervous system. (Dwek et al. 2002) Three clinical subtypes of Gaucher's disease are described. The adult or type I Gaucher's is a non-cerebral form of storage disease, with some residual enzymatic activity, that accounts for its emphasis on splenic, hepatic, and skeletal involvement. Infantile Gaucher's (type II) is an acute disease dominated by cerebral accumulation of substrate, as well as hepatosplenic involvement. No enzyme activity is usually detected. Type III Gaucher's is a juvenile disease intermediate in signs and symptoms from types I (adult) and II (infantile). The involvement of the CNS and pre-existing skeletal muscle lesions has significant impact on the efficacy of enzyme replacement therapy.

The disease pathology results directly from either CNS dysfunction, or the physical effects of the accumulation of distended macrophage "Gaucher's cells" in spleen, liver, marrow, lymph nodes, thymus, and Peyer's patches. These long-term effects include anemia and thrombocytopenia. Longitudinal studies with 2–5 years of clinical follow-up have been published in type I Gaucher's disease, using recombinant glucocerebrosidase targeted to macrophage lysosomes through the endocytic route (Weinreb et al. 2002). Enzyme replacement is highly effective in ameliorating extra-CNS disease manifestations. Patients show long-term improvements and maintenance of improvement in anemia and thrombocytopenia. There is also a measurable benefit in bone erosions and bone pain, with more than 50% of patients showing measurable improvement even after the presence of radiologically documented bone lesions and bone pain (Bembi et al. 2002; Poll et al. 2002). This is a pleasing and rational approach to the correction of a primary heritable peripheral defect of macrophages, and illustrates the therapeutic accessibility of macrophages that have access to the circulation. Large molecules equilibrate inefficiently across the blood–brain barrier in the absence of specific transcytotic mechanisms or leak-

age, and therefore the primary CNS defect is not amenable to approach via the circulation, and will likely require the development of effective gene therapies or alternate approaches to the modification of glycosylation pathways (Dwek et al. 2002). The disease, because of its low prevalence, will always be considered an orphan. This ultimately limits the resources that could be brought to therapeutic approaches, and reflects that market forces will always favor the highly prevalent, and patients and physicians will need to rely upon small companies seeking market niches, or academic institutions with governmental or charitable funding to approach these diseases.

3
TNF Sequestrant Therapy Improves the Outcome of RA and Sheds Critical Mechanistic Insights into Crohn's Disease

The central role of the macrophage in the mechanism of human disease has emerged from the clinical evaluation of TNF sequestrants in rheumatoid arthritis and Crohn's disease. The impact of the elucidation of the pathophysiology of TNF on therapeutics, sheds light into the long lag phase between the leading edge of macrophage biology and its therapeutic application. TNF-α was discovered in the 1970s by Old and colleagues (Feldmann and Maini 2001) and cloned in the early 1980s, when it was shown that cells of the monocytic lineage were the major source of TNF production (Tracey and Cerami 1994). TNF is a rapidly produced proinflammatory cytokine, with serum levels detectable within 30 min of lipopolysaccharide (LPS) stimulus, and likely reflects the cleavage of preformed membrane-bound TNF-α by TNF-α-converting enzyme (TACE). Blockade of TNF release blunts the release of other proinflammatory cytokines such as interleukin (IL)-1 and IL-6, suggesting that TNF plays a role as a molecular trip wire for the activation of stress responses to noxious stimuli, that engage a cascade of events culminating in spatially and temporally regulated recruitment of inflammatory and immune leukocytes at sites of injury. In keeping with these data was the demonstration of the pivotal role of TNF-α in the early control of intracellular bacterial pathogens. The work of Havell in listeriosis (Havell 1989) and Vassalli (Kindler et al. 1989) in early granuloma formation and the control of Bacillus Calmette-Guerin (BCG) infection in mice, both accurately showed the role of TNF-α as an essential organizer of the granulomatous response, and foreshadowed the efficacy of TNF sequestration in Crohn's disease, (Sandborn and Targan 2002) as well as the deleterious potentiation of bacterial diseases including tuberculosis (Tb) by TNF sequestration. This remains one of the few areas of basic biology where early studies have so accurately prospectively predicted the adverse effects expected upon clinical usage. The lag phase between characterization of the role of TNF and the central role of the macrophage as organizer and participant in the granuloma, and the clinical exploitation of these data was almost 15 years. The features that contributed to the gap between the basic discovery and the clinical exploitation included developing adequate methods of production and manufacture, as well as the lengthy requirements

for clinical trials in chronic diseases with complex clinical endpoints such as rheumatoid arthritis (Feldmann and Maini 2001; Bresnihan 2002; Jenkins and Hardy 2002; Kalden 2002; Scott 2002; Weisman 2002) and Crohn's disease. In addition, the essential role of TNF in host defense coupled with the long half-lives of TNF sequestrants required significant empirical approaches to dose ranging. The choice of dose in attempting to validate a pharmacological mechanism is always difficult, in this case especially so because of the need to walk a tightrope between suppression of the host response sufficient for efficacy, and suppression of macrophage function to the point where host defense impairment results in catastrophic potentiation of rapid bacterial infections. The advantage of the rheumatoid arthritis field is that standard therapies including methotrexate and corticosteroids also have well-documented risks of potentiation of infection. With appropriate patient exclusion criteria, the clinical safety of TNF sequestration has been acceptable, with a somewhat higher prevalence of upper respiratory infection in First World usage. Recent reports have included the reactivation of miliary Tb in patients by TNF sequestrants, and more than 70 reports of Tb reactivation can now be found within the Adverse Experience database maintained by the Food and Drug Administration (FDA) (Keane et al. 2001; Mayordomo et al. 2002).

An unexpected clinical finding has been the development of demyelinating disease in a small number of patients receiving TNF sequestrant therapy for adult or juvenile rheumatoid arthritis (Sicotte and Voskuhl 2001). Trials of lenercept in multiple sclerosis (Lenercept 1999) showed that there were no significant differences between groups on any magnetic resonance imaging (MRI) study measure, but the number of lenercept-treated patients experiencing exacerbations was significantly increased compared with patients receiving placebo (p=0.007) and their exacerbations occurred earlier (p=0.006). These findings suggest that one of the roles of TNF may be in the control of demyelination, and that the macrophage, by extension, can play pleiotropic roles in human pathology, exacerbating rheumatoid arthritis and Crohn's disease, while playing a suppressive role in multiple sclerosis. Another hint towards a suppressive role for TNF in disease is the small incidence of drug-induced lupus on TNF sequestrant therapy. This again is in keeping with the predictive value of basic studies, where TNF-α deletant mice also developed elevated levels of anti-double stranded (DS) DNA antibodies (Ettinger and Daniel 2000).

The findings that TNF sequestration can have beneficial effects upon the expression of Crohn's disease but not ulcerative colitis, has again sharpened the focus on the role of the macrophage in Crohn's disease, and strongly differentiated the pathogenesis of Crohn's disease from ulcerative colitis (Podolsky 2002; Sandborn and Targan 2002). One of the strengths of the pharmacological approach is this ability, through controlled clinical trials, to provide insights into human as opposed to model diseases. It is, however, salutary that in this field at least, disease models in lower species including rodents have in large part been prospectively predictive of both efficacy and adverse experience.

4
Bisphosphonates Enhance Bone Mass Retention and Protect Against Fractures by Inhibition of Osteoclast Formation

Whereas the two previous examples deal with macrophage enzyme replacement using recombinant enzyme, or sequestration of a macrophage-secretory product TNF with antibodies or receptor fusion proteins, the efficacy of small synthetic chemical moieties, specifically nitrogen-containing bisphosphonates, impact upon the resorption of bone mass, by inducing osteoclast and macrophage apoptosis.

Bisphosphonates are the most effective inhibitors of bone resorption and are extensively used for the treatment of systemic or local bone loss including postmenopausal osteoporosis and tumor bone disease. Bisphosphonates are pyrophosphate analogs, which bind to bone. Bisphosphonates are then removed from the mineral matrix in the acidic compartment formed between the osteoclast and the bone surface, analogous to the sealed compartments described in macrophages upon immune complexes or complement ligands (Wright and Silverstein 1984). Labeled bisphosphonates accumulate in osteoclasts and inhibit further bone resorption (Reszka et al. 1999). Evidence has accumulated that all bisphosphonates that inhibit the resorption of bone induce the caspase-dependent formation of pyknotic nuclei and the cleavage of Mst1 kinase. This cleavage of Mst1 kinase and caspase activation is dependent upon the bisphosphonate inhibition of the mevalonate pathway, and is specifically blocked by the addition of geranylgeraniol, a key precursor for geranylgeranyl diphosphate (Benford et al. 1999; Reszka et al. 1999). It emerges from these studies that the flux through the mevalonate pathway to geranylgeraniol is essential for the formation of osteoclasts from macrophages, and for the long-term maintenance of the osteoclast population (Fisher et al. 1999; Fisher et al. 2000). In the absence of protein geranylgeranylation, osteoclasts fail to differentiate from macrophages in murine, rabbit, and chicken systems, and that this failure can be attributed to the effects of bisphosphonates on geranylgeranylation and not farnesylation of proteins (Coxon et al. 2000). The effects of bisphosphonates are therefore complex. Effective pharmacodynamic inhibition of bone resorption that can be measured both as the retention of bone mass, as well as by the inhibition of fractures in long-term clinical use (Karpf et al. 1997) can be achieved by the modulation of osteoclast differentiation from macrophages, as well as by the inhibition of osteoclast function through the activation of caspase-mediated apoptotic events.

5
Future Directions

These areas have shown the therapeutic feasibility of macrophage enzyme replacement, secretory product sequestration, and inhibition of osteoclast differentiation and function. Efficacious therapies with direct effects upon macro-

phages now target rheumatoid arthritis, Crohn's disease, osteoporosis, tumor disease of bone, and inherited metabolic defects of the mononuclear phagocyte system. The important role of the macrophage as a regulator and remodeler of tissue, and regulator of the migratory and differentiative events of tissue cells has not yet been exploited. That the macrophage plays a central role in atherosclerosis is clear. The therapies effectively directed towards the role of the macrophage in that disease still requires reduction to practice. It may be that many of the therapeutically advantageous effects of peroxisome proliferator-activated receptors (PPAR) agonists in vascular disease will prove to be useful probes of macrophage function in human pathology (Berger and Moller 2002).

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Transcription Factors That Regulate Macrophage Development and Function

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Abstract The transcription factors that play a major role in the development and function of the monocyte/macrophage lineage are outlined in this review. Hematopoiesis proceeds through the binding of specific combinations of transcription factors leading to a temporal and lineage-restricted pattern of gene expression. We summarize current knowledge of how transcription factors are able to drive monocyte differentiation from early pluripotent progenitors. These transcription factors include numerous homeobox family proteins, AML-1, Pu.1, MZF-1, Egr-1, ICSBP, and STAT family proteins. The transcription factors that control inducible gene expression in mature macrophages are also covered. Macrophages are able to respond to a range of bacterial products and play a critical role in both the innate and adaptive immune response to infection. The inducible transcription factors that regulate inflammatory and anti-microbial gene products covered here include NF-κB/Rel, IRF, STAT, and C/EBP family proteins. This activation of macrophages must be precisely regulated to prevent damage to host tissue. Many transcription factors can act as repressors of gene expression in macrophages and the signals that down-regulate inflammatory responses such as activation of TGFβ, PPARγ and glucocorticoid receptors are discussed. Macrophages play a fundamental role in adaptive immunity and are likely to influence the formation of either a Th1 or Th2 pattern of immune reac-

tivity. We briefly review the current understanding of how activation of different transcription factors can influence the profile of cytokines expressed in macrophages and contribute to the formation of distinct immune responses.

Keywords Gene regulation, Transcription factors, Macrophage development, Inflammation, Immune response

1 Introduction

The mononuclear phagocyte family is present throughout development and in every organ system, where they can comprise 10%–15% of the total cell mass. Progenitor cells in the bone marrow give rise to blood monocytes that can enter tissue and further develop into distinct tissue macrophages. Macrophages are vital to both the innate and acquired immune response by direct endocytosis and cytotoxicity, antigen presentation, and production of biologically active molecules such as cytokines and chemokines. Their destructive potential underlies many aspects of the pathology of acute and chronic inflammation.

Macrophages share many constitutively expressed genes, such as the endocytic receptors responsible for recognition of microorganisms. The expression of other genes is acutely regulated by external stimuli such as microbial products, lymphokines, and growth factors. Ultimately, the pattern of gene expression is determined by specific transcription factors. This chapter deals with the control of transcription in cells of the macrophage lineage.

2 Transcription Factors in Macrophage Development

Hematopoiesis branches into distinct pathways through a temporal and lineage-specific pattern of transcription factor expression. Lineage commitment appears to take place through rapid changes in the expression or function of specific transcription factors. This pattern of activation may arise extrinsically, from activation by growth factors or intrinsically, from either genetically coded or stochastic events. Lineage commitment can still occur in the absence of many cytokines known to influence myelopoiesis. Although the hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-3 and granulocyte macrophage (GM)-CSF can regulate growth and differentiation from bone marrow precursors, they do not appear to be required for steady-state hematopoiesis (Nishinakamura et al. 1995). In contrast, macrophage (M)-CSF (CSF-1) is required for macrophage production as determined by the phenotype of mice with a targeted deletion of the CSF-1 receptor or a natural mutation in the CSF-1 gene (Cecchini et al. 1997; Dai et al. 2002).

Molecular events involved in early myelopoiesis have been extensively studied due to their importance in the formation of myeloid leukemias. Most studies

have focused on either transcription factors that are targets for gene translocation in leukemia or interacting proteins that act in concert to effect proliferation or differentiation of progenitor cells. Leukemia-associated proteins include several members of the homeobox superfamily of genes, acute myeloid leukemia protein-1 (AML-1), promyelocytic leukemia zinc finger protein (PLZF) and myeloid zinc finger protein (MZF-1). Many transcription factors that effect myeloid development are also required for gene expression in differentiated macrophages. These proteins include the Ets protein family transcription factor PU.1, the bZIP proteins of the CCAAT enhancer binding protein family (C/EBP), c-Maf, AML-1 and the zinc finger proteins, specificity protein 1 (Sp1) and the early growth response factor (Egr)-1. A number of transcription factors can respond to the extrinsic stimuli that regulate myelopoiesis. Retinoic acid receptors (RAR) are important in induction of terminal granulocyte differentiation and signal transducers and activators of transcription (STAT) transcription factors control cytokine-induced differentiation. This review will briefly describe early events in myeloid development with emphasis on transcription factors associated with monocyte/macrophage development.

2.1 Early Myeloid Development

One of the most common targets for translocation in acute myeloid leukemia is within the gene that encodes the AML-1 (Runx1) transcription factor. Many translocations disrupt AML-1 function by formation of a fusion protein that replaces the *trans*-activation domain with a histone deacetylase repressor protein (ETO, MTG16) (Hiebert et al. 2001). Interaction between AML-1 and the cofactor, CBFβ allows high-affinity binding to DNA and forms the core binding factor (CBF) protein complex (Bushweller 2000). AML-1 and CBFβ are normally expressed in all hematopoietic tissues during myeloid differentiation and in mature macrophages (Tracey and Speck 2000). The CBF protein complex appears to act as a master regulator of hematopoiesis since targeted deletion of either AML-1 or CBFβ in transgenic mice resulted in a complete loss of fetal liver hematopoiesis (Lutterbach and Hiebert 2000). CBF is thought to act as an organizing factor to facilitate the actions of other transcription factors. AML-1 is able to bind to C/EBP proteins and PU.1, and these interactions are important for expression of CSF-1 receptor on myeloid progenitors and mature macrophages (Petrovick et al. 1998). C/EBP proteins and PU.1 can both synergistically activate the human CSF-1 receptor promoter with AML-1 (Zhang et al. 1996a). Like many of the transcription factors mentioned here, AML-1 may lie downstream of hematopoietic growth factor signaling pathways. CSF-1 receptor signaling activates the ras-raf-MAPK pathway that leads to serine phosphorylation of AML-1 by the extra-cellular signal regulated kinase-1 (ERK1). ERK-dependent phosphorylation potentiates the *trans*-activation ability of AML-1 and may be important for differentiation of progenitor cells and activation of mature macrophages (Tanaka et al. 1996).

The function of the *HOX* family of homeobox genes in hematopoiesis was also identified through chromosomal abnormalities associated with certain leukemias. These *HOX* genes are expressed in somewhat lineage-restricted patterns and are an important component of the coded pattern of gene expression during hematopoiesis. There is evidence that HOXA10, HOXA9, HOXA5, HOXB3, HOXB8, and HOXB7 can influence myeloid development (Lawrence et al. 1997). Targeted deletion of HOXA9 resulted in approximately 30% reduction in total leukocytes and lymphocytes, accompanied by atrophy of the spleen and thymus. Myeloid/erythroid and pre-B progenitors in the marrow were significantly reduced with little or no disruption of earlier progenitors (Lawrence et al. 1997). Overexpression of HOXB3 in bone marrow cells resulted in loss of nearly all B-cell progenitors, with elevated numbers of granulocyte-macrophage colony forming cells in the spleen and bone marrow (Sauvageau et al. 1997). HOXA5 may also regulate lineage restriction. HOXA5 expression in CD34($^+$) multipotent progenitors shifted differentiation toward myelopoiesis and away from erythropoiesis (Crooks et al. 1999). Down-regulation of HOXA10 may be required to allow cells to progress to later stages of myeloid development (Thorsteinsdottir et al. 1997). Targeted deletion of HOXA10 resulted in a twofold increase in peripheral blood neutrophils and monocytes and a fivefold increase in myeloid progenitors (Tenen et al. 1997). The expression of HOXA10 is highest in CD34$^+$ progenitors and is down-regulated during myeloid development (Lawrence et al. 1995).

Some HOX proteins appear to specifically promote monocyte development, but this activity may be a result of ectopic expression. Further analysis will be required to determine if these effects are consistent with the normal pattern of expression during hematopoiesis. HOXB8 expression in the 32Dcl3 progenitor cell line inhibited granulocyte differentiation in response to G-CSF but was required for GM-CSF induced monocyte differentiation (Krishnaraju et al. 1997). Similarly, HOXB7 expression in the HL60 cell line inhibited retinoic acid-induced granulocyte differentiation but not vitamin D3-induced monocyte differentiation (Lill et al. 1995). It should be noted that HOXB7 does not appear to be expressed in monocytes and was not induced by treatment with CSF-1 (Lill et al. 1995).

Several transcription factors from the zinc finger family are potentially important in myeloid development. These proteins contain cysteine and histidine residues that bind zinc ions to form a protein loop capable of binding a range of specific DNA sequences. Zinc finger proteins involved in myeloid differentiation belong to the *Drosophila Kruppel*-related proteins and include Sp1, MZF-1, and Egr-1.

MZF-1 transcription factors appear to be important for maintenance of myeloid progenitor cells. MZF-1 expression was detected in the myeloid lineage from myeloblasts to metamyelocytes and not in other bone marrow cell types (Bavisotto et al. 1991). Consistent with this expression pattern, the MZF-1 promoter contains binding sites for MZF-1, PU.1, and retinoic acid receptors (Hui et al. 1995). Expression of MZF-1 in the IL-3-dependent myeloid cell line,

FDCP1, decreased apoptosis after withdrawal of IL-3 and led to tumor formation in 70% of transduced cells. Similarly, MZF-1 decreased retinoic acid-induced apoptosis in HL-60 cells (Hromas et al. 1996; Robertson et al. 1998). The neoplastic and anti-apoptotic characteristics of MZF-1 suggest it may function to expand numbers of myeloid precursors before they proceed to terminally differentiate.

The Sp1 and Egr-1 transcription factors are important regulators of both basal and inducible gene expression in mature macrophages and are discussed in more detail later. Although they do not show lineage-restricted expression, there is significant evidence that both can play a limited role in monocyte development. A number of studies have shown that Sp1 can mediate specificity and inducibility during cell differentiation. There is evidence of enhanced Sp1 binding to sites in myeloid cells possibly due to cell-specific phosphorylation of Sp1 (Chen et al. 1993; Zhang et al. 1994a,b). Egr-1 is an early response gene that can be induced by a variety of stimuli and is up-regulated during monocytic but not granulocytic differentiation. Expression and anti-sense inhibition of Egr-1 provided evidence that Egr-1 acts as a specific inducer of monocyte differentiation (Nguyen et al. 1993; Krishnaraju et al. 1995; Lee et al. 1996). Disruption of the Egr-1 gene in transgenic mice failed to show any abnormality in monocyte development, but this result may be due to compensation by the other Egr-1 family members, Egr-2 and Egr-3 (Lee et al. 1996).

The STAT transcription factors are a family of seven proteins important for cytokine-regulated gene expression (Ward et al. 1999). STAT transcription factors are latent until tyrosine phosphorylated by receptor-associated Janus kinases (JAK) (Parganas et al. 1998). STAT proteins are important for induction of genes during immune responses and this activity will be discussed later in the review. STAT3 plays a crucial role in proliferation and survival of many cell types and targeted deletion of STAT3 is embryonic lethal (Takeda et al. 1997). IL-6 induced macrophage differentiation of the M1 cell line required STAT3 activation (Hirano et al. 2000). STAT5a and STAT5b can both contribute to myeloid cell development by mediating activation through GM-CSF signaling. Targeted deletion of both STAT5a and STAT5b, among other defects, showed a decrease in colony forming ability in response to GM-CSF (Teglund et al. 1998). Both STAT3 and STAT5 are able to activate important proliferation and anti-apoptosis genes and are a common target for viral oncogenes such as *v-abl* (Kieslinger et al. 2000; Nosaka et al. 1999). STAT5 proteins are likely to act on myeloid progenitors by enhancing proliferation and cell survival. Signaling through the CSF-1 receptor is able to activate STAT1, STAT3 and STAT5 and activation of STAT1 and STAT3 is augmented by co-stimulation with γ-interferon (γ-IFN) (Novak et al. 1996). Since CSF-1 receptor expression is low in progenitor cells and increases as cells differentiate to monocyte/macrophages, induction of STAT proteins could be critical for proliferation, survival and differentiation of macrophages in response to CSF-1 (Eilers and Decker 1995; Eilers et al. 1995).

2.2 Monocyte Differentiation

The mechanism that allows myelopoiesis to branch into the monocytic and granulocytic pathways is not well defined. One theory is that the level of specific transcriptional activators determines the pathway of cell development. Many of these transcription factors regulate their own expression, and small changes in expression may push cells towards lineage commitment. The transcription factors most closely tied to monocyte development are PU.1, interferon consensus sequence binding protein (ICSBP or IRF-8), and c-Maf.

PU.1 is a member of the Ets family of transcription factors and is the product of the *Spi*-1 oncogene identified in Friend virus-induced erythroleukemias. PU.1 is expressed at low levels during early hematopoietic development and specifically up-regulated during myeloid development (Cheng et al. 1996). PU.1 is expressed at high levels in myeloid and B cells but is not found in T cells (Chen et al. 1995a). This expression pattern suggests that PU.1 can play a role in commitment to early myeloid lineages as well as the later stages of differentiation. Consistent with this role is the down-regulation of PU.1 during erythropoiesis and the inhibition of erythropoiesis observed upon retroviral overexpression in bone marrow cultures (Schuetze et al. 1993). Gene knockout of PU.1 in mice gave conflicting results. One knockout resulted in embryonic lethality at day E16 (Scott et al. 1994) and another resulted in viable animals that could be kept alive for days under sterile conditions (Mckercher et al. 1996). Although the first knockout suggested PU.1 disruption could affect early multipotent progenitors, the second demonstrated a more restricted phenotype, consistent with the pattern of PU.1 expression. Recent research has demonstrated that this difference in transgenic animals is due to the genetic background of the mice, suggesting that other transcription factors can compensate to a varying extent for PU.1 deficiency (Luchin et al. 2001). Viable transgenic animals showed an absence of monocytes and mature B cells but were still capable of producing B-cell progenitors. T cells and neutrophilic cells were present although neutrophils were reduced in number and altered in function (Mckercher et al. 1996; Anderson et al. 1998).

The PU.1 protein contains an 80 amino acid DNA-binding domain, characteristic of Ets factors, located at the carboxyl terminal end (Klemsz et al. 1990). Like other Ets factors, PU.1 binds to purine-rich sequences characterized by a loose consensus core motif GGAA (Klemsz et al. 1990), but binding is distinct from the other Ets family members, Ets-1, Ets-2, Elf-1, and Fli-1 (Voso et al. 1994). The PU.1 gene promoter itself contains a PU.1-binding site important for function in myeloid cells. A significant up-regulation of PU.1 coincides with the first detection of early myeloid progenitors, suggesting amplification of PU.1 occurs through an autocrine loop and this enhanced expression may play a major role in commitment to the myeloid lineage (Voso et al. 1994; Chen et al. 1995a). A model of how PU.1 can act as an essential component of monocyte develop-

ment yet play a more restricted role in B-lymphocyte and granulocyte development is emerging.

At one level, PU.1 expression is much higher in macrophages than B cells and over-expression of PU.1 in B cells can actually suppress activity of B cell-specific enhancers (Ross et al. 1994; Dekoter and Singh 2000). Recent research in myeloid development has focused on proteins that interact with PU.1 or compete for DNA binding, and these two mechanisms also contribute to the restricted activity of PU.1 in the formation of certain cell types. The Ets domain of PU.1 can interact with the C-terminal finger region of the transcription factor GATA-1, leading to repression of PU.1-mediated gene expression (Nerlov et al. 2000). GATA-1 is down-regulated in myeloid development but plays an important role in development of non-myeloid lineages (Voso et al. 1994). In a similar manner, B cell-specific activator protein (BSAP) PAX5 can interact with PU.1 through the *trans*-activation domain and inhibit PU.1 activity (Maitra and Atchison 2000). The expression of BSAP is restricted to B lymphopoiesis. Another lymphocyte-specific inhibitor of PU.1 is a second Ets-family protein, PU.1-related factor (Prf), that can compete for DNA binding. Prf is expressed during B-lymphocyte development only and since Prf does not appear to function as a transcriptional activator, it may function as an antagonist of PU.1 activity (Hashimoto et al. 1999).

These mechanisms suggest that PU.1 activity is of limited importance in early B-lymphocyte development. Although PU.1 is required for final maturation and expression of genes in differentiated B lymphocytes, the role of PU.1 during lymphopoiesis is less defined. The reduction of PU.1 activity during B lymphopoiesis would also be expected to have a minimal impact due to partial redundancy with the closely related Spi-B transcription factor (Garrett-Sinha et al. 2001). Spi-B is expressed in B lymphocytes and not in myeloid cells and Spi-B gene knockout in PU.1 +/− mice resulted in a reduction of immature and mature B lymphocytes (Chen et al. 1995b; Su et al. 1996).

The ability of PU.1 to interact with members of the interferon regulatory factor (IRF) family may also be significant in directing monocyte differentiation. The IRF family member, ICSBP, has been shown to regulate myeloid differentiation (Holtschke et al. 1996; Scheller et al. 1999; Tamura et al. 2000). The IRF family members are able to bind the interferon-stimulated response elements (ISRE) found in many interferon-inducible genes (Contursi et al. 2000). ICSBP is expressed in monocyte/macrophage lineages, B lymphocytes, and activated T lymphocytes and is induced by γ-interferon (IFN) (Darnell et al. 1994). Although γ-IFN alone is myelosuppressive, in combination with CSF-1 or GM-CSF it is an activator of monocytopoiesis (Breen et al. 1991). ICSBP forms a complex with PU.1 and activates composite elements in the myeloid-specific gp91(phox) p67(phox) and Toll-like receptor 4 genes (Eklund and Kakar 1999; Rehli et al. 2000). Targeted deletion of the ICSBP gene resulted in a significant increase in granulocytes and a reduction in mature macrophages with a significant impairment of the ability of GM-CSF or CSF-1 to form macrophage colonies (Scheller et al. 1999). Expression of ICSBP in a bipotential cell line, derived from ICSBP$^{-/-}$ mice, showed that

ICSBP drives myeloid progenitors to differentiate into macrophages but inhibits granulocyte differentiation (Tamura et al. 2000).

The PU.1 gene knockout described above also proposed a limited role for PU.1 in early granulocyte development. Similar to B cells, PU.1 appeared to be significant mainly in the final stages of granulocyte differentiation. This stage-specific role is consistent with considerable evidence that commitment to the granulocyte lineage is closely associated with expression of C/EBP proteins and not PU.1 (Gombart and Koeffler 2002). The C/EBP family is a subfamily of bZIP (leucine zipper) proteins. Relevant C/EBP family members include C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε, all of which bind to similar DNA sequences (Antonson et al. 1996). Targeted deletion of either the C/EBPα or C/EBPε genes resulted in severe impairment of granulocyte development (Yamanaka et al. 1997a; Zhang et al. 1997). Expression of both C/EBPα and C/EBPε increases during granulocyte differentiation but decreases during monocyte differentiation (Morosetti et al. 1997; Scott et al. 1992; Yamanaka et al. 1997b). Interestingly, the ectopic expression of C/EBPε alone in 32Dcl3 progenitor cells was sufficient for differentiation to mature granulocytes, indicating a certain level of functional redundancy (Khanna-Gupta et al. 2001).

This expression pattern is not true for a third member of the family, C/EBPβ (NF-IL6) which increases during monocyte but not granulocyte differentiation (Natsuka et al. 1992). In the mouse, C/EBPβ is a strong transactivator of the CSF-1 receptor (*c-fms*) gene promoter (Xie et al. 2002). C/EBPβ shows low activity in myeloid cells until cells are activated by inflammatory mediators and is generally associated with inducible gene expression that is discussed later in the review. Gene knockout of C/EBPβ resulted in no abnormalities in myeloid differentiation, although there were deficiencies in macrophage activation at later stages (Screpanti et al. 1995). The possibility that C/EBP family members can substitute in pairs has not been eliminated.

Another bZIP family member that affects monocyte development is c-Maf. Overexpression of c-Maf induces HL60 and U937 cells to terminally differentiate to macrophages (Hegde et al. 1999). c-Maf is thought to promote macrophage differentiation by forming complexes with c-Myb and inhibiting specific c-Myb regulated targets (Hedge et al. 1998). Gene knockout of c-Myb resulted in embryonic lethality and a failure of fetal liver hematopoiesis (Mucenski et al. 1991). c-Myb is thought to act early in hematopoiesis, and its expression is down-regulated during differentiation (Gewirtz and Calabretta 1988). This down-regulation may be important for monocyte differentiation since c-Myb has been shown to inhibit the CSF-1 receptor gene (Reddy et al. 1994). Inhibition of c-Myb activity appears to be of lesser importance for granulocyte development. c-Myb can synergize with C/EBP proteins and a number of granulocyte-specific genes are *trans*-activated by c-Myb (Oelgeschlager et al. 1996; Verbeek et al. 1999).

The microphthalmia transcription factor (MiTF) family is a set of four related members of the bHLH-ZIP class of proteins. The MiTF family member is required for development of osteoclast cells, a closely related cell lineage to mac-

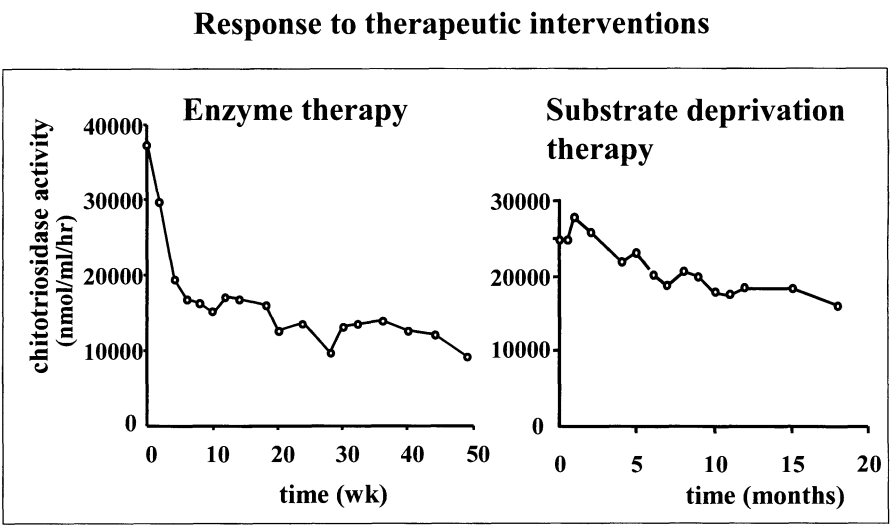
rophages (Moore 1995). Evidence of their role in monocytopoiesis arises from the dominant phenotype of a subset of MiTF mutations. MiTF transcription factors bind DNA as homodimers or heterodimers with other family members and there is evidence that MiTF proteins interact with PU.1 (Steingrimsson et al. 1994; Luchin et al. 2001). Targeted deletion of individual MiTF family members was not associated with any deficiency in macrophage development; however, this probably reflects the redundant expression in macrophages of all four members, MiTF, TFE-3, TFE-B, and the myeloid-specific member, TFE-C (Rehli et al. 1999).

3
Common Regulatory Elements in Macrophage-Specific Promoters

Regulation of macrophage-specific genes requires the coordinate assembly of multiple transcription factors onto distinct regulatory elements. Promoters that direct lineage-specific expression in macrophages are different from many other tissue-specific promoters. Macrophage-specific promoters generally lack a defined initiator sequence, such as a TATAA box. Transcription initiation can occur at multiple sites within the promoter or at a single strong start site. Transfection of tissue culture cells showed that macrophage restricted expression in some genes required only a small region upstream of the major transcription start site. Deletional analysis of the PU.1 and human CSF-1R promoters showed that minimal, tissue-specific promoter activity was encoded in an approximately 90-bp region (Zhang et al. 1994a; Chen et al. 1995a; Kistler et al. 1995; Ross et al. 1998). The core promoter elements that allow transcription initiation in the absence of a TATAA box are a major component of macrophage-restricted gene expression as outlined below.

Several transcription factors have been shown to interact with TATAA-binding protein (TBP), and this interaction may allow certain transcription factor binding sites to substitute for a TATAA sequence. The presence of functional Sp1 sites in the initiation sequence of many promoters may overcome the absence of a TATAA sequence. Sp1 has been shown to interact with a number of proteins that influence TFIID binding and hence transcription initiation. Sp1 can interact with TBP as well as TAF110 and CRSP, a cofactor complex that can directly bind TFIID (Emili et al. 1994; Ryu et al. 1999). Although Sp1 expression is ubiquitous, the DNA-binding activity can be cell-type specific as outlined above. An in vivo study showed that Sp1 binds the CD11b promoter specifically in myeloid cells and was required for myeloid-specific promoter activity (Chen et al. 1993).

Many macrophage-specific promoters, however, do not contain Sp1 sites within the core upstream promoter sequence. These promoters show a consistent pattern of elements near the start of transcription as outlined in Fig. 1. The purine-rich elements within these regions have been repeatedly identified as PU.1 binding sites. Indeed, nearly all of the macrophage-specific promoters analyzed so far contain a PU.1 site near the start of transcription. An interesting ex-



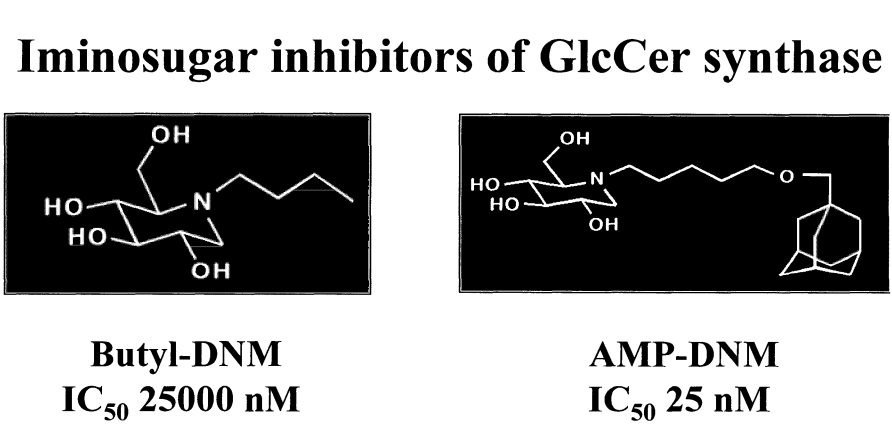


Fig. 1 Sequence comparison of the proximal promoter regions of macrophage-restricted genes. Sequences are aligned by an E box-like motif present in many macrophage-restricted promoters lacking a TATAA box element. *Arrows* mark published transcription initiation sites. PU.1/Ets-like GGAA motifs are shaded in *gray* and *dark shading* indicates motifs where mutation resulted in a significant loss of myeloid-specific promoter activity

ception is the CD14 promoter, which contains a TATAA box initiation site but no PU.1 binding site (Zhang et al. 1994b). The CD14 promoter is capable of directing cell-specific expression only in transient assays and not in transgenic mice (Ferrero et al. 1993). Similarly, the chicken lysozyme gene also lacks a PU.1 site in its promoter but contains one in an upstream enhancer important for cell-restricted expression (Ahne and Stratling 1994). PU.1 probably acts to recruit the basal transcription complex. A purine-rich repeat alone can function as a macrophage-specific promoter (Ross et al. 1998) and PU.1 has been shown

to bind directly to TBP in vitro (Hagemeier et al. 1993). Further evidence that PU.1 can play a role in macrophage-specific transcription initiation was provided by study of Fcγreceptor1 gene expression. Basal and γ-INF-induced expression was abolished by mutation of the PU.1 site, and replacement of the mutated site with a TATAA box restored activity (Eichbaum et al. 1994).

Other Ets family transcription factors are likely to be important for macrophage promoter activity. Macrophage-specific genes typically contain several purine-rich sequence elements, not all of which bind PU.1. The other Ets factors expressed in myeloid cells are Ets-2, Fli-1, Elf-1, and myeloid Elf-1-like factor (MEF) (Klemsz et al. 1993; Kola et al. 1993; Voso et al. 1994). The Ets-2 transcription factor mediates activation of genes in response to CSF-1 receptor signaling such as urokinase plasminogen activator (υPA) and scavenger receptor (Fowles et al. 1998). Activation of the ras-raf-MEK1-ERK kinase pathway by CSF-1 results in phosphorylation of Ets-2 and increased transcriptional activity (Fowles et al. 1998). Ets-2 has also been shown to *trans*-activate the CSF-1 receptor promoter (Ross et al. 1998). Targeted deletion of Ets-2 was embryonic lethal at E8.5, but other studies showed Ets-2 can play an important role in basal and inducible gene expression in macrophages (Reddy et al. 1994; Henkel et al. 1996; Yang et al. 1996).

Binding sites for C/EBP transcription factors are also common in myeloid-specific promoters. Although C/EBP proteins are critical for granulocyte development, there is evidence for a role in macrophage gene expression. The C/EBPβ family member is active in macrophages and is important for inducible gene expression. Targeted deletion of C/EBPβ resulted in impaired macrophage function and mice were immunodeficient (Screpanti et al. 1995). As mentioned above, the level of C/EBPβ specifically increased during monocyte differentiation and C/EBPβ is activated by phosphorylation with ERK kinases and hence a target for CSF-1 receptor signaling. Although clearly not required for lineage development, C/EBPβ can *trans*-activate the CSF-1 receptor promoter with AML-1 and PU.1. It is not clear how this interaction, perhaps in response to infection, can potentiate cell-specific gene expression or differentiation of macrophages (Zhang et al. 1996b; Xie et al. 2002).

Many of the protein complexes that bind regulatory elements in macrophage-specific genes are still not identified. A comprehensive analysis of at least one macrophage-specific gene may be required to catalogue required transcription factors. Advances in array technologies such as gene chips may allow identification of transcription factors and the pathways that regulate them. Changes in chromatin structure are an important determinant of the pattern of gene expression and many transcription factors recruit co-factors that can acetylate or deacetylate histones. Studies on how transcription factors restructure chromatin will also be needed to understand the complex architecture of protein binding that enables cell-specific expression.

4
Regulation of Inducible Gene Expression

Regulation of inducible gene expression in macrophages is of crucial importance to the pathogenesis of several diseases including inflammation, septic shock, atherosclerosis, rheumatoid arthritis, pulmonary fibrosis, and inflammatory bowel disease. During the course of infection, the activation of macrophages must be strictly controlled to prevent damage to host cells. The precise regulation of macrophages is also required to direct host defenses towards specific pathogens and sites of infection. Adaptive immunity has been loosely categorized into two distinct T-cell responses, based on the profile of cytokines expressed. The response of T helper (Th)-1 cells is mainly directed towards systemic infection by viruses and bacteria, and Th2 cell responses are directed towards mucosal infections, primarily in response to infection by parasites. Macrophage or T-cell activation by two distinct subsets of cytokines directs these responses. Autocrine or paracrine stimulation with IL-4 or IL-10 promotes Th2 cell responses and stimulation with IL-12 or γ-IFN promotes Th1 cell responses (Schulze-Koops and Kalden 2001). The role of macrophages in the formation of these two T cell subsets is generally understated. Macrophages are the most sensitive cell type to the microbial products that activate distinct immune responses. For example, the ability of bacterial DNA to specifically potentiate the Th1 immune response is well established yet T cells are not capable of responding to bacterial DNA (Weiner 2000). Additionally, macrophages develop into distinct lineages in tissue and are likely to mediate organ-associated immune responses such as activation of distinct T cell subsets or immune tolerance in the intestinal mucosa. This review will concentrate on recent studies of transcription factors that control gene expression in response to pro-inflammatory and anti-inflammatory signals.

4.1
Pro-inflammatory Signals

NF-κB/Rel Proteins. Nuclear factor-κB/Rel proteins are a small family of transcription factors that are latent in the cytoplasm by virtue of their association with IκB inhibitor proteins. A large number of extracellular signals can trigger a variety of distinct signal transduction pathways that lead to the degradation of IκB. The nuclear localization signal on NF-κB/Rel proteins is then unmasked, allowing translocation to the nucleus and DNA binding as hetero or homodimer protein complexes. NF-κB/Rel proteins are important for the induction of nearly all of the proteins associated with inflammation (Silverman and Maniatis 2001). The ubiquitination and proteolytic degradation of IκBs is activated by the IκB kinases, IKK$\alpha/\beta/\gamma$ (Cheng et al. 1996; Lee and Rikihisa 1998; Silverman and Maniatis 2001). Although activated by similar signals, the NF-κB/Rel family proteins possess distinct functions. The p50 and p52 members do not contain the C-terminal *trans*-activation domain, and *trans*-activation requires dimerization

with the p65 (RelA), RelB, or c-Rel proteins (Silverman and Maniatis 2001). The prototypic protein complex that mediates inducible gene expression is the NF-κB, p50/p65 heterodimer. The primary activation signals in macrophages are microbial products such as lipopolysaccharide (LPS) and bacterial DNA, IL-1, IL-6, and tumor necrosis factor-α (TNF-α) (Zhang and Ghosh 2000). The mechanisms of signal transduction and activation of NF-κB/Rel is not covered here, since they have been reviewed elsewhere (Silverman and Maniatis 2001).

Several studies have shown that transcriptional activation by Rel proteins required both the degradation of IκB and Rel protein phosphorylation and that these two activation signals can be uncoupled (Schmitz et al. 2001). NF-κB, p65 has a protein kinase A phosphorylation site on serine 276 (Zhang et al. 1997). Phosphorylation at this site was dependent on IκB degradation and was required for binding to the transcriptional co-activator, CREB-binding protein (CBP) (Zhong et al. 1998). A second site on p65, serine 529, was phosphorylated in response to TNF-α stimulation and was also dependent on IκB degradation (Wang and Baldwin 1998; Wang et al. 2000a). Phosphorylation of s529 appeared to be mediated by casein kinase II (Wang et al. 2000a) and resulted in enhanced transcriptional activation but not nuclear translocation or DNA binding. IL-1 has also been shown to induce phosphorylation of p65, and this phosphorylation required phosphotidylinositol-3 kinase and Akt (Sizemore et al. 1999). This signaling appears to involve IKKβ and required serines 529 and 536 of p65 (Madrid et al. 2001). PI3 K and Akt stimulation was again found to enhance transcriptional activity and not nuclear translocation or DNA binding (Madrid et al. 2001). Considerably less work has been performed on activation of c-Rel proteins; however, many of the phosphorylation sites in p65 occur in the *rel* homology domain. There is also evidence that uncoupling of IκB degradation and transcriptional activation occurs with c-Rel. Targeted deletion of c-Rel resulted in enhanced expression of GM-CSF, IL-6, and TNF-α in peritoneal macrophages treated with LPS but reduced expression of GM-CSF and IL-2 in activated T cells (Gerondakis et al. 1996).

The evidence that s276 phosphorylation allowed binding to the co-activator CBP may in part explain the anti-inflammatory activity of PPARγ signaling and cAMP activation. Both PPARγ, discussed below, and cAMP-response element binding protein (CREB) may compete for limiting amounts of CBP and interfere with p65 transcriptional activation (Li et al. 2000). This activity highlights the potential therapeutic value of compounds that target the phosphorylation of p65 or the associated binding proteins during inflammation, particularly since targeted deletion of p65 or IκB produced a lethal phenotype (Silverman and Maniatis 2001).

STAT Proteins. Many cytokines that are important for cellular activation in the macrophage lineage utilize the JAK-STAT signaling pathway. The ligand-activated γ-IFN receptor recruits JAK1 and JAK2 kinases, leading to phosphorylation, nuclear translocation, and binding of STAT1 homodimers to GAS (γ-IFN activated site) elements. The activation of STAT proteins is transient and several

constitutive and inducible pathways down-modulate STAT activity (Darnell 1997). Constitutive pathways that down-modulate STAT activity include dephosphorylation, proteolytic degradation, and binding to inhibitor molecules (Haspel et al. 1996; Kim and Maniatis 1996; Chung et al. 1997a,b). Induction of the suppressors of cytokine signaling molecules (SOCS) by a large number of cytokines is important for feedback inhibition of JAK kinase activity and allows cross-inhibition between cytokine receptors (Endo et al. 1997; Starr et al. 1997). Signaling through the mitogen-activated protein kinase (MAPK) pathway has also been shown to inhibit STAT activation (Sengupta et al. 1998). Phorbol ester, ionomycin or GM-CSF activation of ERK kinase has been shown to inhibit IL-6-mediated JAK1 and JAK2 phosphorylation of STAT3 (Petricoin et al. 1996; Sengupta et al. 1996). Other pathways that inhibit STAT activation in macrophages include, crosslinking of Fc or complement CR3 receptors (Feldman et al. 1995; Marth and Kelsall 1997) and activation of the signal transducers, protein kinase A and protein kinase C (Bhat et al. 1995; Lee and Rikihisa 1998). STAT proteins are also regulated through serine phosphorylation by kinases yet to be identified. The functional significance of serine phosphorylation varies and can either activate or inhibit tyrosine phosphorylation or DNA binding (Eilers et al. 1995; Wen et al. 1995; Beadling et al. 1996).

The precise regulation of STAT proteins in macrophages may be instrumental in directing either Th1 or Th2 cell responses. For example, the cytokine IL-10 is a potent growth factor for activated B cells, but down-modulates T-cell responses by suppressing expression of major histocompatibility complex class II and B7 on macrophages (Ding et al. 1993). The induction of SOCS3 by IL-10 can block γ-IFN mediated STAT1 activation by binding to phosphorylated residues in the tyrosine kinase domain of JAK kinases (Ito et al. 1999). STAT1 along with IRFs are required for expression of IL-12, an important cytokine for directing Th1 responses (Durbin et al. 2000). Strong inflammatory signals from bacteria can also alter cell signaling. Treatment of macrophages with LPS, IL-1, and TNF-α inhibited IL-6 and IL-10 but not γ-IFN activation of STAT phosphorylation and DNA binding (Ahmed and Ivashkiv 2000).

IL-4 can induce expression of a distinct subset of genes in macrophages, including Fcεreceptor IIb (CD23), 15-lipoxygenase, and IL-1 receptor antagonist (Vercelli et al. 1988; Conrad et al. 1992; Fenton et al. 1992). The induction of STAT6 by IL-4 signaling is important in both activation and inhibition of gene expression (Kaplan et al. 1996; Shimoda et al. 1996; Takeda et al. 1996). Pretreatment of macrophages with γ-IFN or β-IFN can suppress activation of STAT6 by IL-4. This repression is mediated by interferon-induced expression of the JAK–STAT inhibitor SOCS1 (Dickensheets et al. 1999). IL-4 activation of STAT6 binding can, in turn, suppress γ-IFN-induced transcription. STAT6 appears to inhibit interferon-induced gene expression by competition for occupancy of promoter binding sites with STAT1 (Ohmori and Hamilton 1998). STAT6$^{-/-}$ mice showed decreased susceptibility to septic peritonitis due to enhanced bacterial clearance. This enhanced anti-microbial activity was associated with in-

creased levels of IL-12, TNF-α, and macrophage-derived chemokine (Matsukawa et al. 2001).

IRF Proteins IRF Protein. IRFs are a family of at least nine transcription factors with a broad range of activities (Taniguchi et al. 2001). All members are expressed in most cell types except IRF-4 and ICSBP, which are expressed in lymphoid and myeloid cells only (Driggers et al. 1990; Rosenbauer et al. 1999). The members that effect macrophage activation the most are IRF-1, interferon-stimulated gene factor-3γ (ISGF3γ, IRF-9), and ICSBP (IRF-8). IRF-1 expression is strongly enhanced in macrophages by treatment with γ-IFN (Flodstrom and Eizirik 1997). Targeted disruption of IRF-1 resulted in a wide range of defects in macrophage activation and consequent resistance to several models of chronic inflammatory disease. Macrophages from IRF$^{-/-}$ mice failed to induce iNOS (inducible nitric oxide synthetase) in response to LPS or β-IFN (Kamijo et al. 1994; Martin et al. 1994). The iNOS enzyme catalyzes production of nitric oxide, important for intracellular killing of pathogens and may explain the severe pathology of *Mycobacterium bovis* infection in IRF-1$^{-/-}$ mice (Kamijo et al. 1994). IRF-1 was also required for expression of the p40 subunit if IL-12, essential for activation of the Th1-cell subset in the immune system (Lohoff et al. 1997).

ICSBP (IRF-8) shows selective expression in myeloid and lymphoid cells and is important for macrophage development as discussed above. ICSBP is induced by γ-IFN but not by α-IFN or β-IFN (Kanno et al. 1993). ICSBP can bind to composite elements by formation of activating transcriptional complexes with IRF-1, IRF-2, and PU.1 (Eklund et al. 1998). Transcriptional activation by ICSBP is critical for the Th1 immune response since ICSBP$^{-/-}$ mice failed to produce IL-12 and γ-IFN and showed increased susceptibility to virus infection (Wang et al. 2000b).

ISGF3γ/IRF-9 also forms activating complexes upon stimulation with α and β-IFNs and exerts its transcriptional effects exclusively by association with STAT1 and STAT2 (Veals et al. 1992; Bluyssen et al. 1996; Darnell 1997). The resulting trimolecular IRF-9/STAT1,2 complex, referred to as ISGF3, is able to activate transcription of many genes by binding to ISREs (Stark et al. 1998). γ-IFN treatment of macrophages can strongly enhance transcriptional activation through ISREs by up-regulating IRF-9 and STAT1 expression. There is also evidence that γ-IFN together with spontaneously produced α- or β-IFN allows physical association between the IFN receptors and STAT1/2 docking and activation (Bandyopadhyay et al. 1990; Levy et al. 1990).

C/EBP ProteinsC/EBP Protein. The principle C/EBP family members that activate gene expression in response to inflammatory stimuli are C/EBPβ and C/EBPδ. The level of C/EBPε is extremely low in mature macrophages and C/EBPα levels generally decrease in response to inflammatory signals (Tengku-Muhammad et al. 2000). C/EBPβ is predominantly expressed in monocyte/macrophages, hepatocytes, keratinocytes, and adipocytes and is the principle mediator of C/EBP-induced inflammatory gene expression in macrophages (Akira et

al. 1990; Cao et al. 1991; Lekstrom-Himes and Xanthopoulos 1998; Maytin and Habener 1998). The expression and activity of C/EBPβ is induced by many inflammatory mediators such as LPS, IL-1, IL-6, γ-IFN, and TNF-α (Akira et al. 1990; Pope et al. 1994; Darville and Eizirik 2001; Hu et al. 2001). γ-IFN can stimulate the transcriptional activity of C/EBPβ through activation of ERK1 and ERK2 kinases. This phosphorylation and activation of C/EBPβ was required for induction of ISGF3γ/IRF-9 expression, allowing formation of the ISGF3 complex on ISREs (Hu et al. 2001). Induction of C/EBPβ plays a central role in acute-phase responses by activating expression of a number of inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-8, MIP-1α, MCP-1, and MMP-1 (Grove and Plumb 1993; Shirakawa et al. 1993; Stein and Baldwin 1993; Pope et al. 1994). A role for C/EBP proteins has been established in a number of inflammatory diseases (Poli 1998). Macrophages that express high levels of C/EBPβ were found in the synovial lining of patients with rheumatoid arthritis and the level of expression strongly correlated to lining thickness (Pope et al. 1999).

Egr-1 ProteinsEgr-1 Protein. A number of studies have shown that Egr-1 transcription factors are important for macrophage activation during chronic or acute inflammation (Mcmahon and Monroe 1996). Many recent studies have specifically analyzed the role of Egr-1 in the pathogenesis of atherosclerotic lesions (Khachigian 2001). Egr-1 is induced not only by inflammatory cytokines and LPS, but also by enzymatically degraded low-density lipoprotein (LDL), hypoxia, physical force, and injury (Silverman and Collins 1999). Egr-1 activation influences expression of many stress-response genes including platelet-derived growth factor (PDGF), transforming growth factor (TGF)β, TNF-α, intracellular adhesion molecule (ICAM)-1, urokinase-type plasminogen activator, and metalloproteinases (Mccaffrey et al. 2000). Elevated levels of Egr-1 can be found at early stages of atherosclerosis with progressive increase in expression during formation of lesions, particularly in areas of macrophage infiltration (Mccaffrey et al. 2000). Activation of Egr-1 may be important for infiltration of macrophage into lesions. Egr-1 expression and binding was required for activation of the CD44 promoter in response to IL-1 (Maltzman et al. 1996). Expression of the adhesion molecule, CD44 is important for recruitment of leukocytes to inflammatory sites, and CD44 signaling results in expression of pro-inflammatory chemokines in macrophages (Ariel et al. 2000; Stoop et al. 2001).

4.2 Anti-inflammatory Signals

SMAD ProteinsSMAD Protein. The cytokine TGFβ can act as a potent anti-inflammatory agent in macrophages, and up-regulation of TGFβ in gastrointestinal tissue is essential for induction of oral tolerance (Letterio and Roberts 1998). Targeted deletion of the TGFβ1 gene resulted in systemic inflammation and early death (Shull et al. 1992). SMADs are a family of proteins that transduce signals from type I and type II TGFβ receptors (Massague and Wotton

2000). TGFβ activation of the type I receptor results in serine phosphorylation of SMAD2 and SMAD3 which associates with SMAD4 during nuclear transport forming complexes that bind DNA and recruit transcription factors (Letterio and Roberts 1998; Massague and Wotton 2000). Inhibition of LPS activation in macrophage by TGFβ treatment required SMAD3 and appeared to involve the ability of SMAD3 to compete for the co-activator p300 (Werner et al. 2000).

The pro-inflammatory cytokines, TNF-α and γ-IFN can both inhibit the TGFβ/SMAD signaling pathway. These cytokines inhibit SMAD activity by inducing the expression of the repressor, SMAD7 that can occupy ligand activated TGFβ1 R1 and block SMAD2 and SMAD3 phosphorylation (Hayashi et al. 1997; Nakao et al. 1997). Disruption of the SMAD signaling pathway is implicated in the pathogenesis of inflammatory bowel disease. SMAD7 expression was up-regulated in mucosal tissue from patients with Crohn's disease and ulcerative colitis, and analysis of lamina propria mononuclear cells from diseased tissue showed extremely low levels of phosphorylated SMAD3 and these cells did not respond to TGFβ treatment (Monteleone et al. 2001).

PPARγ. Recent research has provided evidence that the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors can act as a down-modulator of inflammation. PPAR proteins are capable of both positive and negative regulation of gene expression in response to ligand binding. PPAR proteins contain a highly conserved DNA-binding domain and ligand-dependent and -independent *trans*-activation domains (Nolte et al. 1998; Xu et al. 1999). PPAR positively regulates gene expression by binding as a complex with retinoid X receptors to composite elements within target genes (Direnzo et al. 1997). The PPARγ family member is expressed in macrophages and appears to be involved in lipid accumulation and inflammatory responses. Both the natural ligand, 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 and the thiazolidinedione (TZD) class of synthetic ligands have anti-inflammatory effects on macrophages and TZDs show early signs of efficacy against inflammatory bowel disease (Delerive et al. 2001; Lewis et al. 2001). Treatment of macrophages with PPARγ ligands reduced expression of the inflammatory cytokines TNF-α, IL-1β and IL-6 (Jiang et al. 1998). Treatment of elicited macrophages induced a resting phenotype and suppressed iNOS, gelatinase B and scavenger receptor A (Ricote et al. 1998). PPARγ inhibition of the iNOS promoter did not involve DNA binding. PPARγ appears to inhibit gene expression by suppressing the activity of NF-κB, AP-1, and STAT1 transcription factors through competition for recruitment of the shared co-activators p300 and CBP (Ricote et al. 1998; Li et al. 2000). IL-4-mediated inhibition of iNOS synthesis partially involves PPARγ by inducing the coordinate expression of PPARγ and its ligands 13 HODE and 15 HETE (Ricote et al. 2000).

Evidence that PPARγ may influence lipid accumulation in macrophages has led to concerns over the use of TZDs in the treatment of diabetes. PPARγ-activated THP-1 cells showed increased expression of the scavenger receptor CD36 and increased uptake of oxidized LDL (Chawla et al. 2001). Targeted deletion and retroviral expression of PPARγ confirmed that CD36 expression is enhanced

by PPARγ (Chawla et al. 2001). Other reports, however, have shown that PPARγ does not promote formation of macrophage foamy cells and can activate cholesterol efflux by induction of apo A1-mediated cholesterol transporter (ABCA1) gene expression (Chinetti et al. 2001).

Glucocorticoid ReceptorGlucocorticoid Receptor Activation. Glucocorticoids (GC) are used as immunosuppressive agents in organ transplantation, immune diseases, and inflammatory disorders. GCs suppress many functions in macrophages, the most significant of which is inhibition of cytokine production, including suppression of TNF-α production. Administration of GCs results in adverse effects in many tissues and causes systemic monocytopenia in humans (Joyce et al. 1997). GCs have been shown to interfere with the function of members of the NF-κB and AP-1 family of transcription factors (Auphan et al. 1995). The decreased binding of NF-κB seen with glucocorticoid treatment may result from either interaction between ligand-activated glucocorticoid receptor and p65/RelA or induction of IκB synthesis (Ramdas and Harmon 1998; Costas et al. 2000). Recent evidence suggests that glucocorticoids can alter the function of histone acetylases by inhibiting p65-associated acetylase transferase or CBP recruitment by NF-κB (Ito et al. 2001; Kagoshima et al. 2001). There is, however, evidence that post-transcriptional mechanisms contribute significantly to glucocorticoid inhibition of cytokine production. In humans, glucocorticoids reduce the stability of IL-1β, IL-1α, and IL-6 mRNA (Keffer et al. 1991; Amano et al. 1993).

GCs also have a profound effect on the regulation of Th1/Th2 cytokine responses (Almawi et al. 1999). Treatment with the GC, dexamethasone resulted in specific inhibition of IL-12 activation of STAT4 with no effect on IL-4 activation of STAT6 (Franchimont et al. 2000). The loss of IL-12 activity resulted in reduced γ-IFN expression from T cells and natural killer cells and a subsequent decrease in the ability of macrophages to direct Th1 cell responses. The resulting Th2 cytokine profile with GC treatment is believed to be a result of this inhibition of Th1 cell responses. GCs were not shown to directly enhance Th2 cytokine responses and addition of γ-IFN abrogated the dexamethasone enhanced Th2 cytokine profile (Agarwal and Marshall 2001; Miyaura and Iwata 2002).

5 Future Studies

Macrophage-specific gene expression involves the temporal and lineage-specific assembly of transcription factor complexes on DNA. A critical step in transcription factor access to DNA is the remodeling of chromatin, and both cell differentiation and macrophage activation require extensive changes in chromatin structure. Many developmental transcription factors such as NF-κB, AML-1, homeobox family proteins, and HMG-box proteins can modify chromatin either directly or by binding specific co-factors. Identifying the DNA-binding proteins that allow rapid changes in chromatin structure is an important area for future

study. Finally, it is clear that a large number of important transcription factors are still not identified. A detailed analysis of protein binding has been performed on a few select promoters. Even within these genes, the transcription factors that bind many of the conserved elements have not been identified. These studies may require technology that allows rapid identification of proteins assembled on promoter or enhancer sequences. Recent advances in microchip technology, used for analysis of protein–protein interaction, may help to identify proteins that associate with transcription factors or structural proteins in chromatin. Expression profiling by microarray can determine the spectrum of transcription factors present during macrophage development and activation. These analyses may reveal new targets for novel therapeutics that act at the level of gene transcription.

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Exploitation of Macrophage Clearance Functions In Vivo

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Abstract The propensity of macrophages for the phagocytic clearance of colloidal particles provides a rational approach to macrophage-specific targeting and drug delivery. Furthermore, by precision engineering, colloidal drug carriers can be targeted to selective population of macrophages in the body as well as intracellular locations. These approaches have led to the development of a number of regulatory-approved particulate formulations for delivery of therapeutic and diagnostic agents to macrophages. This article will briefly discuss selected approaches and highlight barriers in in vivo macrophage-specific targeting with colloidal carriers via intravenous, subcutaneous and oral routes of administration, and it explores avenues for selective modification of macrophage cellular activity.

Keywords Antigen presentation, Colloidal carriers, Drug delivery, Kupffer cells, Liposomes, Lymph nodes, M cells, Macrophage suicide, Nanospheres, Poloxamer, Poloxamine, Splenic macrophages, Stimulated macrophages

Abbreviations

APC	antigen-presenting cell
EO	ethylene oxide
IES	interendothelial cell slits
MHC	major histocompatibility complex
PO	propylene oxide
TAT	trans-activating transcriptional activator

1 Introduction

Macrophages are widely distributed throughout the body and perform a wide range of homeostatic, physiological, and immunological functions. Among them, phagocytosis (or endocytosis) is the macrophage's primary task, which has been well conserved throughout evolution. This is achieved by virtue of a vast array of specialised plasma membrane recognition receptors with which the macrophage can arrest and eliminate senescent and damaged cells, particulate debris and microbial invaders (Gordon 1995).

The propensity of macrophages for the phagocytic/macropinocytic clearance of foreign particles provides a rational approach to macrophage-specific targeting through suitable particulate vehicles. The concept of particulate targeting of macrophages is an attractive one, in that a wide variety of systems are available and particles with differing physicochemical properties and loading capacities can be constructed (Poznansky and Juliano 1984; Moghimi et al. 2001). The particulate entities which have been used include liposomes, niosomes, polymeric nanospheres, oil-in-water microemulsions, and even natural constructs such as lipoproteins and erythrocytes. For targeting, three criteria must be considered. The first criterion is the distribution of macrophages in tissues in terms of access from various physiological portals of entry. The second involves determinants of phagocytic recognition and ingestion, which includes macrophage phagocytic/endocytic receptors (e.g. the nature, density, and their state of activation) and the effect of environmental factors on their phagocytic functions. The last is the physicochemical characteristics of the particles to be ingested and includes particle morphology, hydrodynamic size and surface characteristics (e.g. ligand expression, bound opsonins). This article will briefly discuss selected approaches and highlight barriers in in vivo macrophage-specific targeting with colloidal carriers via intravenous, subcutaneous and oral routes of administration and explores avenues for selective modification of macrophage cellular activity.

2 The Macrophage as a Therapeutic Target for Nanocarriers

Colloidal targeting of macrophages offers a number of advantages. These include treatment of diseases and disorders of the macrophage system as well as attempts

to manipulate phagocytic cell number and their functional activities. For example, although most micro-organisms are killed by macrophages, many pathogenic organisms have developed means for resisting macrophage destruction following phagocytosis. In certain cases, the macrophage lysosome and/or cytoplasm is the obligate intracellular home of the micro-organism, examples include *Toxoplasma gondii*, various species of *Leishmania*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Listeria monocytogenes*, and *Cryptococcus neoformans* (Alving 1988; Donowitz 1994). The targeting of antimicrobial agents encapsulated in colloidal vehicles to infected macrophages is therefore a logical strategy for effective microbial killing. The endocytic pathway will direct a colloidal carrier to lysosomes where pathogens are resident. Degradation of the carrier by lysosomal enzymes can release drug into the phagosome-lysosome vesicle itself or into the cytoplasm either by diffusion or by specific transporters, depending on the physicochemical nature of the drug molecules (Alving 1988; Lloyd 2000). On the other hand, delivery to cytosol can be further enhanced by triggering drug release in late endosomes. Examples include pH-sensitive and fusogenic vesicles (Drummond et al. 2000). A pH-sensitive vesicle maintains stable phospholipid bilayers at neutral pH or above but destabilises and becomes fusion-competent at the acidic pH of late endosomes and subsequently release their encapsulated cargo into cytoplasm (Horwitz et al. 1980). Alternatively, by mimicking the characteristics of certain toxins or viruses, cytoplasmic delivery of agents can also be enhanced. This requires co-encapsulation of bacterial pore-forming toxins (e.g. listeriolysin O) or fusion peptides found in envelope glycoproteins of certain viruses (e.g. HA2 fusion peptide of influenza virus) in particulate vehicles (Lee et al. 1996; Beauregard et al. 1997; Drummond et al. 2000). The fusion glycoprotein of such viruses displays sharp fusion profiles with pH midpoints from 5.0 to 6.6, which is in good agreement with estimates of the endosomal pH. These targeting strategies also overcome the toxicity of drugs effective against microbes. For example, the most prominent potential toxicities associated with pentavalent antimonials (e.g. sodium stibogluconate) used for leishmaniasis treatment include changes in the electrocardiogram and hepatocellular damage. Thus direct targeting allows significant reductions in the required quantity of drugs, while achieving therapeutic drug concentrations in the infected cell.

Macrophages play an important role in induction of immunity. The induction of an immune response against a protein antigen invokes the interaction of the antigen with macrophage [or an antigen-presenting cell (APC)] that partially degrades the antigen and channels peptides into the MHC molecules (class I or class II) for processing and presentation (Fling et al. 1994; Harding and Song 1994; Kovacsovics-Bankowski and Rock 1995). These highly polymorphic MHC class I and class II molecules bind and transport peptide fragments of intact proteins to the surface of APCs for interaction with either $CD4^+$ or $CD8^+$ T lymphocytes. It is generally accepted that endogenous proteins of a cell are presented via the MHC class I pathway, whereas exogenous peptides are presented via the MHC class II route. Most soluble antigens are poor at priming MHC class I-restricted cytotoxic T-lymphocyte responses because of their inability to gain access to the

cytosol. Colloidal carriers and particularly liposomes, however, act as powerful adjuvants if they are physically associated with a protein antigen (Gregoriadis 1990; Rao and Alving 2000). After phagocytosis by macrophages or APCs, the entrapped antigens in liposomes are presented to either MHC class I or class II pathways. Successful cytotoxic T-cell responses may further be obtained following antigen delivery with pH-sensitive liposomes as the antigen cargo may be delivered either to group 1 CD1 molecules (which also belong to MHC class I molecules but are localised in the endosomes and are able to bind and present glycolipids and microbial lipids) or be released directly into the cytosol.

Phagocytosis is not a prerequisite for intracellular targeting. Decoration of the nanoparticulate surface with *trans*-activating transcriptional activator (TAT) protein from HIV-1, or related peptides, facilitates cytoplasmic entry, which does not involve endocytosis and occurs by an energy-independent process (Torchilin et al. 2001). So far, TAT protein movement and import do not appear to be cell-specific. Although this approach may have implications for intracellular delivery of biologicals, the intracellular fate of the carrier must also be considered. A related technology is based on the remarkable cellular trafficking properties of the 35-kDa herpesvirus-1 structural protein VP22 (Elliott and O'Hare 1997). This protein reaches the nuclear compartment, despite lacking a nuclear localisation sequence, and binds chromatin in a matter of minutes. It also can act as a soluble carrier to transport peptides and proteins to the cell nucleus and therefore it is an attractive candidate for delivery of transcriptional factors, functional genes, cell cycle control regulators and DNA vaccines to macrophages and stem cells in vitro prior to transplantation.

There are many potential dysfunctions of macrophages that may be involved directly or indirectly in pathogenesis of diseases. For example, newborn infants manifest increased susceptibility to lung infections due to deficiency in alveolar macrophage-mediated secretion of biological response modifiers (Lee et al. 2001). Other examples include autoimmune blood disorders, spinal cord injury, sciatic nerve injury, T cell-mediated autoimmune diabetes and rheumatoid arthritis (Alves-Rosa et al. 2000; Barrera et al. 2000; Liu et al. 2000; Wu et al. 2000). These conditions should be amenable to treatment or become manageable following challenge with particulate carriers containing encapsulated drugs and genes via appropriate routes of administration. Indeed, colloidal-mediated macrophage suicide (i.e. delivery of macrophage toxins) has proved to be a powerful approach in removing unwanted macrophages in various experimental situations (van Rooijen and Sanders 1997; Alves-Rosa et al. 2000; Barrera et al. 2000; Liu et al. 2000; Wu et al. 2000; Kotter et al. 2001; Polfliet et al. 2001).

Macrophages are also heterogeneous with respect to phenotype and physiological properties, even within a single organ (Gordon et al. 1992; Rutherford et al. 1993). Understanding of macrophage heterogeneity will undoubtedly provide new insights and opportunities for designing carriers that can selectively deliver agents to defined macrophage sub-populations in vivo (see also "Targeting and Therapeutics: Colloid Engineering Meets Immunobiology"). An interesting attempt is to enhance delivery to newly proliferated macrophages or newly re-

cruited monocytes (Moghimi and Patel 2002) at selected sites rather than local resident macrophages.

Apart from therapeutic goals, colloidal carriers are also useful for assessing macrophage phagocytic and clearance functions in vivo. Similarly, particulate colloids with entrapped radiopharmaceutical or contrast agents are helpful in imaging a designated pathology through macrophage loading (Tilcock 1995; Moghimi and Bonnemain 1999).

3
Targeting and Therapeutics: Colloid Engineering Meets Immunobiology

3.1
Intravenous Route

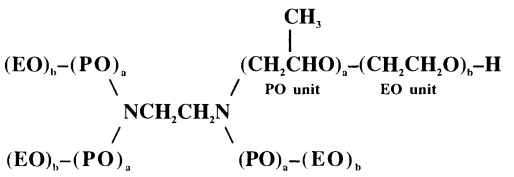
3.1.1
Resident Macrophages in Contact with Blood

Hepatic phagocytes or Kupffer cells are the largest population of macrophages in contact with blood (approximately 90%) and are the most prominent participants in particle clearance from the circulation (Moghimi et al. 2001). In addition to Kupffer cells, splenic marginal zone and red pulp macrophages also participate considerably in particle sequestration from the blood. Particle extraction by splenic macrophages is further enhanced in phagocytic malfunctions of Kupffer cells; this is simply due to increased blood concentration of particles (Moghimi et al. 2001).

Advantage has been taken of the natural physiological fate of particulate drug carriers to deliver, albeit passively, a variety of agents, which include antimicrobials and immunomodulators, to Kupffer cells and splenic macrophages (Poznansky and Juliano 1984). This specific delivery has resulted in direct killing of microbes and in the activation of the phagocytes to a bactericidal, fungicidal and tumouricidal state. The therapeutic efficacy of particulate-encapsulated drug may be further improved by targeting of the carrier to specific macrophage plasma membrane receptors. One such example is tuftsin receptor. Tuftsin, a part of the Fc portion of the heavy chain of the leukophilic IgG, is a natural macrophage-activator peptide. The binding of tuftsin to its receptor causes macrophage activation. Intravenous injection of tuftsin-bearing liposomes to infected animals not only resulted in delivery of liposome-encapsulated drugs to the macrophage phagolysosomes, but also in the non-specific stimulation of liver and spleen macrophage functions against parasitic, fungal and bacterial infections (Agrawal and Gupta 2000). Another interesting approach for future exploration is surface decoration of liposomes and nanoparticles with mannose receptor ligands, since ligands of mannose receptor carbohydrate recognition domains may modulate macrophage function (Linehan et al. 2001).

Our understanding of splenic architecture and intrasplenic microcirculation has provided us with new opportunities for selective targeting of nanoparticles to splenic marginal zone and red pulp macrophage. To enhance splenic reten-

(A) Structure of Poloxamines:



poloxamine 904	a = 17	b = 15
poloxamine 908	a = 17	b = 119

(B) Structure of Poloxamers:

$(EO)_b$–$(PO)_a$–$(EO)_b$

poloxamer 401	a = 67	b = 5
poloxamer 402	a = 67	b = 11
poloxamer 403	a = 67	b = 20
poloxamer 407	a = 67	b = 98

Fig. 1
Structure of poloxamines (**A**) and poloxamers (**B**)

tion of particles, a number of criteria must be met (Moghimi et al. 1991, 1993b,c; Moghimi and Hunter 2000). First, particles must exhibit prolonged circulation times in the blood; that is to avoid rapid clearance from the blood by Kupffer cells. Second, particles must be non-deformable and their size should be equivalent or exceed the reported width of splenic interendothelial cell slits (IES) in the sinus walls usually in the order of 200–500 nm. Indeed, IES are the sites that retain blood cells and blood-borne particles, depending on their bulk properties, size, sphericity and deformability. Finally, filtered particles must be prone to phagocytosis by the neighbouring macrophages.

Thus, to prolong particle concentration in the blood, polymeric materials such as dextrans, poloxamer 407, poloxamine 908 and poly(ethyleneglycol)-5000 have been used to camouflage particles against Kupffer cell capture (Moghimi et al. 2001; Gbadamosi et al. 2002; Moghimi 2002). For example, poloxamine 908 (Fig. 1) consists of four central hydrophobic blocks, each composed of 17 propylene oxide (PO) units, joined together by an ethylene diamine bridge. Each hydrophobic block is also attached to a relatively hydrophilic chain consisting of 119 ethylene oxide (EO) units. The hydrophilicity of EO chains arises from hydrogen bonding with water molecules. This copolymer can adsorb onto the surface of hydrophobic particles (e.g. polystyrene nanospheres) via its central hydrophobic portion, while the EO flanks extend from the particle surface and provide stability to the particle suspension by repulsion through a steric mechanism of stabilisation involving both enthalpic and entropic contribu-

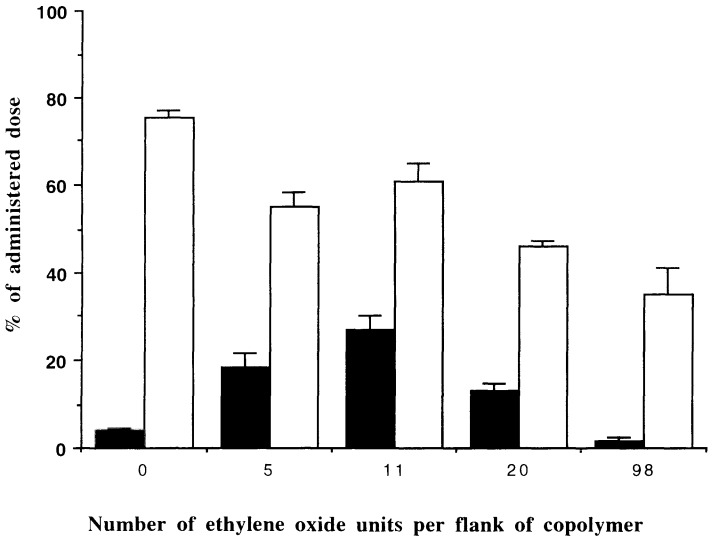


Fig. 2 Footpad and lymph node (popliteal and iliac nodes) distribution of uncoated and poloxamer-coated polystyrene nanospheres (60 nm) at 2 h after subcutaneous injection into rat footpads. Poloxamers 401, 402, 403 and 407 were used for particle coating and their structure is shown in Fig. 1. *Closed columns* represent uptake by regional lymph nodes and *open columns* represent the fraction of particles retained at footpads. For experimental details, see Moghimi et al. (1994)

tions. The steric barrier of EO chains suppresses protein adsorption on to the particle surface as well as particle interaction with plasma membrane of Kupffer cells (Moghimi et al. 1993d). If the size of the camouflaged particles is below 150 nm, they escape splenic filtration at IES and remain in the blood with reported half-lives of 24–48 h (Moghimi et al. 2001). Due to their altered pharmacokinetic properties, these particles can passively accumulate at sites of inflammation, infection and solid tumours, but the extent of particle extravasation is dependent upon the porosity of the blood vessels at such pathological sites. At such sites, activated macrophages can recognise and phagocytose extravasated long circulating particles by an unknown mechanism (Moghimi et al. 2001). On the other hand, if the size of rigid particles is in the range of 200–300 nm, splenic filtration will predominate (in excess of 50% of the injected dose within a few hours of intravenous injection with Kupffer cell uptake of <15% of the dose) (Moghimi et al. 1993c). Surprisingly, in the spleen, filtered poloxamine-coated particles are eventually phagocytosed by the red pulp macrophages; this is presumably due to gradual loss of the surface coating and subsequent opsonisation.

3.1.2
Stimulated or Activated Liver Macrophages

In contrast to resident Kupffer cells, stimulated or activated liver macrophages or newly recruited marginating monocytes can rapidly recognise and ingest

surface-engineered long-circulating as well as poloxamine-based splenotropic particles from the blood by an opsonin-independent mechanism (Moghimi et al. 1993a; Moghimi and Murray 1996; Moghimi and Gray 1997). The nature of macrophage receptors that recognise such engineered particles remains to be unravelled, but a role for CD14, class A scavenger receptors and even Dectins seems possible. Nevertheless, these surface-engineered particles may find applications as diagnostic/imaging tools for stimulated or newly recruited hepatic macrophages (Fig. 2) (Laverman et al. 2001). For example, such diagnostic procedures may prove useful for patient selection or for monitoring the progress of treatment with long-circulating nanoparticles carrying anti-cancer agents, thus minimising damage to hepatic macrophages.

3.2 Subcutaneous Route

3.2.1 Interstitial and Lymph Node Macrophages

The distinct physiological function of the lymphatic capillaries opens up an opportunity for macrophage-specific targeting with colloidal carriers. In these capillaries, numerous endothelial cells overlap extensively at their margins and lack adhesion mechanisms at many points. Immediately following interstitial injection, many of the overlapped endothelial cells are separated and thus passageways are provided between the interstitium and lymphatic lumen, and particles are conveyed to the nodes via the afferent lymph. In lymph nodes, macrophages of medullary sinuses and paracortex are mainly responsible for particle capture from the lymph. However, the behaviour of particles following interstitial administration is controlled by a number of physicochemical and biological factors (Moghimi and Rajabi-Siahboomi 1996).

Physicochemical considerations include particle size and its surface characteristics. Generally, the size of the particles must be larger than 20 nm to prevent their leakage into the blood capillaries. Although larger particles (>100 nm) may carry a considerable amount of agent, they move very slowly from the site of injection into initial lymphatics; the drainage often takes a period of days. This slow transit may induce local inflammation and renders particles susceptible to phagocytosis or macropinocytosis at the injection site (Moghimi et al. 1994). However, considerable differences in drainage and lymph node uptake of particles of similar size can occur. These are explained by differences in surface characteristics of particles, which include the extent of surface hydrophobicity/hydrophilicity and the presence of macrophage-specific ligands. For example, the extent of surface hydrophobicity/hydrophilicity controls particle aggregation at the injection site as well as its interaction with the amorphous ground substance of the interstitium. Hence, the greater the tendency for particle aggregation or interaction with the interstitial structures, the slower the particle drainage and presentation to lymph node macrophages (Moghimi et al. 1994). The

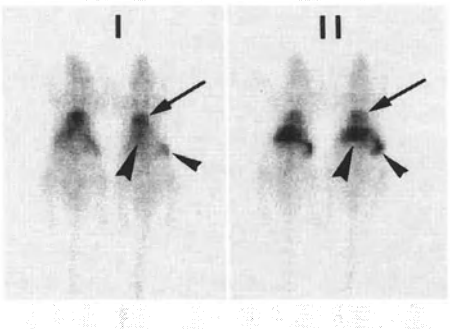


Fig. 3 Scintigraphic images of rats with normal (*I*) and enhanced (*II*) macrophage activity 4 h following intravenous injection of ($^{99m}TcO_4^-$)-labelled long-circulating liposomes. To enhance macrophage phagocytic activity, poloxamine 908 was injected intravenously (43 mg/kg) 3 days prior to liposome injection. In (*I*) the images represent the circulatory blood pool in the heart region (*arrow*) and poor localisation of liposomes in both liver (*large arrowhead*) and spleen (*small arrowhead*) regions. In (*II*) a large fraction of liposomes is captured by stimulated Kupffer cells and splenic macrophages (*arrowheads*)

concept of steric stabilisation of nanoparticles with poloxamer and poloxamine copolymers proved successful in minimising particle retention at the injection site while maximising uptake by local lymph node macrophages (Moghimi et al. 1994). The longer the EO chain (while maintaining the length of central hydrophobic segment) of the coating copolymers, the lesser the tendency for particle aggregation and interaction with interstitial elements. The resulting outcome was rapid particle drainage into initial lymphatics (Fig. 3). The choice of coating copolymer also influences macrophage recognition of particles. For example, the strong steric barrier of poloxamer 407 suppresses particle opsonisation in lymph and/or interaction with macrophage receptors. Such engineered entities drain rapidly, escape clearance by lymph node macrophages, reach the systemic circulation, and remain in the blood for prolonged periods. For medical imaging, these particles have applications for visualising the lymphatic chain, but for macrophage targeting appropriate ligands (e.g. mannose, antibodies) must be attached to the distal end of the hydrophilic chains. To enhance both rapid particle drainage and capture by regional lymph node macrophages simultaneously, particles require coating with copolymers with 5–15 EO units (Fig. 3). These nanoengineering concepts satisfy at least the requirements for lymphoscintigraphy and indirect lymphography (rapid spreading of particles from the injection site and good retention in regional lymph nodes). Other investigators have exploited the adjuvant effect of such engineered nanoparticles in vaccination protocols following interstitial injection as the coating agents (e.g. poloxamers) can manipulate a number of macrophage accessory functions (e.g. upregulation of MHC class II expression) (Hunter and Bennett 1984; Howerton et al. 1990).

Regardless of these surface engineering techniques, particle drainage from the interstitium and their subsequent capture by lymph node macrophages also depends on the potency of the lymphatic system and lymph node. Other biolog-

ical factors which influence particle localisation in lymph nodes include lymph propulsion, obesity and recent surgery in the area of lymphatic drainage and are discussed elsewhere (Moghimi and Rajabi-Siahboomi 1996; Moghimi and Bonnemain 1999).

3.3 The Oral Route

3.3.1 Intestinal Macrophages

A challenging but attractive strategy is to deliver nanoparticles to macrophages in the oral cavity. The majority of the available evidence suggests the adsorption of particulates in the intestine following oral administration takes place at the Peyer's patches (Simecka 1998). The epithelial cell layer overlying the Peyer's patches contains specialised M cells. In the immunocompetent host, the M-cell apical membranes bind and take up bacteria, viruses, inert particles, etc. from the lumen and transport them to underlying macrophages and dendritic cells. The microbial sampling function of M cells is critical to the ability of the mucosal immune system to monitor the contents of the intestinal lumen. Indeed, the use of colloidal particles to deliver vaccines and adjuvants has its foundation in the M cell's ability for transcytosis. Microencapsulation of antigens in such systems also provides better protection for the antigen during intestinal transit. Numerous studies have now confirmed protective immunity induced by mucosal immunisation with polymeric particulate systems (Marx et al. 1993; Jones et al. 1997). The immune outcomes have included mucosal (secretory IgA) and serum antibody (IgG and IgM) responses as well as systemic cytotoxic T lymphocyte responses in splenocytes. Tolerance to orally administered microparticulate encapsulated antigens is another potential outcome but has received little attention. Oral tolerance could provide tremendous potential in treating autoimmune and inflammatory diseases through antigens entrapped in nanoparticles. Low-dose, transmucosal exposure to antigens may also trigger antigen-specific IgE responses (Simecka 1998). T helper-2 cell activation, which supports mucosal IgA responses, promotes isotype switching to IgE-B cells. This is believed to be through secretion of IL-4 (Finkelman et al. 1991). Such IgE responses, whether local or systemic, can mediate potentially life-threatening immune reactions. Thus, IgE responses against antigen should be carefully monitored because of the potential for hypersensitivity reactions in susceptible individuals.

Induction of an appropriate immune response following oral administration depends primarily on factors that affect uptake and particle translocation by M cells. These include particle size, dose, composition and surface chemistry as well as the region of the intestine where particles are taken up, membrane recycling from intracellular sources and the species (Ermak and Giannasca 1998). For example, studies using a number of different microparticles have established that M cells can transport these in a size range similar to that of micro-organisms (re-

viewed by Ermak and Giannasca 1998). A lower limit for size has yet to be determined for M-cell sampling, but the upper limit is believed to be in the region of 10 μm. Following transcytosis particles may be engulfed by phagocytic cells in the dome region or pass through the basal lamina into the sub-endothelial dome region where they gain access via efferent lymphatics to macrophages in mesenteric lymph nodes and peripheral lymphoid organs. Particles transported beyond the follicle-associated epithelium are also disseminated in a size-dependent manner, with dispersion increasing with decreasing particle size (<500 nm). The composition of particles may also affect cytokine production by macrophages and dendritic cells in the dome region. The efficiency of sampling orally delivered nanoparticles by M cells is, however, low as these cells are relatively rare in the epithelial lining (the cumulative surface area of M-cell apical membrane throughout the intestine has been estimated to be 1/10,000th of enterocytes). Therefore, strategies are needed to specifically target the M-cell apical surfaces. Indeed, the use of M cell-selective probes such as secretory IgAs, monoclonal antibodies, lectins, and M-cell attachment proteins of reovirus has enhanced the efficiency of particulate targeting and subsequent presentation to the regional macrophages (Ermak and Giannasca 1998). Such approaches could ultimately be required for optimal stimulation of immunological responses following oral delivery.

4 Current Clinical Trends and Future Prospects

It is now clear that the propensity of macrophages for the phagocytic clearance of colloidal vehicles provides a rational approach to macrophage-specific targeting and drug delivery. To date, this approach has led to the development of a number of regulatory approved particulate formulations. Examples include Ambisome (Gilead Sciences) and a recent liposomal vaccine against hepatitis A. Ambisome is a liposomal formulation of amphotericin B, which is used for treatment of visceral leishmaniasis or confirmed infections caused by specific fungal species.

Alternatively, a significant delay of macrophage involvement would extend the circulation time of intravenously injected colloidal vehicles, thus enabling them to deliver their cargo to non-macrophage sites. Evasion of binding or uptake of particles by macrophages has been achieved by numerous surface modification strategies (Moghimi et al. 2001). Several formulations of stable, long-circulating liposomes are now marketed or seeking approval as carriers of anticancer agents in the treatment of solid tumours. Examples include DaunoXome (daunorubicin, marketed by Gilead Sciences), Doxil (doxorubicin, marketed by Alza Corporation), Onco TCS (vincristine, INEX Pharmaceuticals) and NX211 (lurtotecan, OSI Pharmaceuticals). The next generation of long circulating colloidal carriers are likely to be surface decorated with suitable ligands to internalising receptors overexpressed on tumour cells (e.g. cancers overexpressing HER2/neu proto-oncogene, folate) or angiogenic endothelial cells (e.g. $\alpha_v\beta_3$ integrin complex, Tie-2) (Moghimi et al. 2001). Such approaches will improve the selectivity and anti-tumour activity of existing and newly discovered anti-cancer agents.

Intriguing progress is being made in diagnostic imaging with the development of a long-circulating ultra-small super-paramagnetic iron oxide particle (Sinerem, Guerbet, France). Following intravenous injection, a significant fraction of Sinerem reaches numerous lymph nodes in the body, particularly abdominal and mediastinal nodes, which are the main sites of metastases and are not readily accessible for microscopic evaluation. Interestingly, in the lymph nodes these particles are phagocytosed by resident macrophages. As a result, Sinerem has helped to distinguish between normal and tumour-bearing nodes or reactive and metastatic nodes with magnetic resonance in man (Moghimi and Bonnemain 1999). Sinerem can also be administered subcutaneously.

It is conceivable that future sophistication in colloidal targeting of macrophages as well as non-macrophage cells and the outcome of end-results will depend on the detailed understanding of biological barriers as well as macrophage functions and their recognition mechanisms.

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Reaching the Macrophage: Routes of Delivery

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Abstract The functional heterogeneity of macrophages based on anatomical localization and receptor expression as well as their versatile way through which they react to different stimuli makes them key players in many processes of the body. To stimulate or downregulate their functions in therapeutic ways using their extremely efficient phagocytosis capacity, one has to consider that they are often located at sites that are hard to reach. Local administration of drugs is therefore often the method of choice whereby liposomes seem to be an ideal vehicle through their versatility and relative ease of handling, as well as their efficient uptake by macrophages. Especially the use of the macrophage suicide technique has enabled the study of many functions of the macrophage. An overview is given of the many routes that can be used to reach the macrophage at different sites and the effects this has on the function of the cell.

Keywords Liposomes, Phagocytosis, Clodronate

1 Introduction

Macrophages are extremely versatile cells that have evolved as professional phagocytes. Their phagocytosis capacity is pivotal for the uptake and degradation of infectious agents and senescent or damaged cells of the body. This makes them central cells in tissue remodeling and repair, as well as key players in immune responses and inflammatory reactions.

Although a certain phagocytic capacity can be ascribed to a variety of cells, this capacity is more or less intermediate compared to that of macrophages, and often specialized or restricted as in the case of retinal rod internalization by the epithelial cells of the retina (Rabinovitch 1995).

The extreme efficiency of the macrophage in terms of particle uptake is due to the presence of a vast array of different receptors on the cell membrane that uniquely, or in concert, act to facilitate uptake.

As a consequence of the possible interactions of the various receptors and their signaling modes inside the cell, the process of phagocytosis can be quite complicated and leads to different subsequent actions of the macrophage (Aderem and Underhill 1999).

Many of the receptors on the macrophage that are involved in the binding to pathogenic ligands are able to recognize conserved motifs, so-called pathogen-associated molecular patterns (Janeway 1992). These include mannans found on yeast cell walls, bacterial peptides, lipopolysaccharides, lipoteichoic acid, but also self-molecules that have been modified such as oxidized low-density lipoprotein (LDL) are recognized. The pattern recognition receptors involved form distinct groups based on molecular structure and recognition profile, and they include the family of scavenger receptors, Toll-like receptors, and mannose receptor (Krieger and Herz 1994; Medzhitov and Janeway 2000; Pearson 1996; Peiser et al. 2002; Platt and Gordon 2001). In addition, members of the group of pattern recognition receptors can recognize humoral factors such as components of the complement system, antibodies and factors such as mannan-binding protein. These factors are involved in the opsonization of pathogens, thereby facilitating their uptake by macrophages. The receptors involved in the recognition such as Fc and complement receptors are key players in the phagocytic capacity of macrophages (Carroll 1998; see also Ravetch 1997; Ravetch and Clynes 1998; Stahl and Ezekowitz 1998). Signaling after engagement of these receptors leads to major changes in the actin distribution and mobility of the cell, but can also lead to the production of cytokines and other factors (Wright and Silverstein 1983; Stein and Gordon 1991; Takai et al. 1994; Gerber and Mosser 2001).

2
Heterogeneity of Macrophages

The mode of activation of the macrophage that can occur after engagement of the multiple types of receptors is therefore dependent on the composition of the pathogen or particle to be internalized.

In the case of Fc receptor-mediated uptake of bacteria, strong activation of the macrophage resulting in cytoskeleton rearrangements and the release of pro-inflammatory factors such as tumor necrosis factor (TNF)-α can be seen, whereas the uptake of apoptotic cells, predominantly engaging scavenger receptors and CD14, leads to the production of anti-inflammatory factors such as interleukin (IL)-10 and transforming growth factor (TGF)-β (Savill et al. 1993; Platt et al. 1996; Savill 1998; Fadok et al. 2001). Interestingly, the way the macrophage reacts to stimuli resulting from uptake of different particles and receptor usage is also dependent on the activation state of the cell and may therefore differ considerably (Riches 1995).

In addition, it has become clear that not only the maturation stage of the macrophage in the tissue, from recently immigrated monocyte to fully differentiated macrophage, will determine the potential of the cell to react to external stimuli, but that the tissue itself, where the macrophage is maturing, is of importance for the differentiation of the cell. This leads to major differences in function of macrophages residing, e.g., in the alveoli of the lungs versus the Kupffer cells in the liver (Fathi et al. 2001; Laskin et al. 2001).

Alveolar macrophages, in addition to being efficient scavenging cells, control the homeostasis of the lungs through active downregulation of the activity of T lymphocytes and dendritic cells in the interstitial tissues of the lung (Holt et al. 1993). This capacity is unique for macrophages in the lung microenvironment and seems to be dependent on the production of inhibitory factors by the macrophage, such as nitric oxide. In addition to differences in macrophage populations between different organs, it is clear that even within tissues different subpopulations of macrophages can be discriminated, especially in lymphoid organs such as the spleen. Based on anatomical localization, receptor usage and turnover kinetics, in the spleen at least five different subpopulations of macrophages can be found (van Rooijen et al. 1989a,b; Kraal 1992).

Together with the different modes of activation that the macrophage can develop in response to stimuli, such tissue-dependent specialization adds to the complexity of the cell and forms an important factor when one attempts to manipulate macrophages by specific targeting. Furthermore, the phagocytic capacity of macrophages may lead to unwanted degradation of bioactive products before they can reach their target cells. So, although the efficient uptake of particles by macrophages makes them ideal cells to target, the rapid degradation following phagocytosis may lead to inefficient effects of bioactive materials or unwanted effects on the macrophage.

This can be a problem when macrophages themselves are the target cell, but it also creates an important drawback in attempts to reach other cell types with,

e.g., encapsulated moieties. In the latter cases, such negative influences can be overcome by first killing the macrophages selectively, followed by the administration of the compound that is supposed to activate other cells (van Rooijen et al. 1997; van Rooijen and Sanders 1997).

Furthermore, because of the localization of macrophages in all tissues, often between densely packed cells or collagen fibers, it is not always easy to reach the macrophage. Administration of particulate material into the bloodstream will only reach macrophages that are located in areas where the blood has free access: the blood sinuses of liver and spleen. To reach the macrophage in other tissues, it is necessary to administer locally into the target organ, as is the case for alveolar macrophages (Pantelidis et al. 2001) or skin macrophages (Thepen et al. 2000).

3
How to Reach Macrophages in Various Tissues

The extent to which resident macrophage populations in different organs are accessible to single molecules, molecular complexes or particulate carriers depends on both the position of the macrophages in the tissues and on the properties of the molecules or particles. In general, all macrophages can be reached by small molecules when these are able to pass the capillary networks in order to penetrate into the tissues. For larger molecules, molecular complexes, or particles, the macrophage can only be reached if there is no physical barrier between the site of injection and the macrophage. Such a barrier can be formed, e.g., by endothelial cells in the wall of blood vessels, by alveolar epithelial cells in the lung, by reticular fibers or collagen fibers in the spleen, or by the presence of densely packed cells such as lymphocytes in the white pulp of the spleen or in the paracortical fields of lymph nodes. By choosing the right administration route for the materials to be injected, this barrier can be kept at a minimum.

3.1
The Use of Liposomes

Information on the comparative accessibility of various macrophages in different organs has been obtained from studies using liposomes that lead to the death of the macrophage.

Liposomes (artificially prepared phospholipid vesicles) encapsulating the bisphosphonate clodronate can be used to deplete macrophages in various organs and tissues. Such liposomes, once injected in vivo, will be ingested by macrophages. They are subsequently exposed to lysosomal phospholipases leading to intracellular degradation of the phospholipid bilayers and release of entrapped clodronate molecules within the cells. The more clodronate liposomes will be ingested and digested by the macrophages, the more clodronate will be accumulated within the cells, since it is not easy for these molecules to escape, given their poor ability to cross cell membranes. At a certain intracellular concentra-

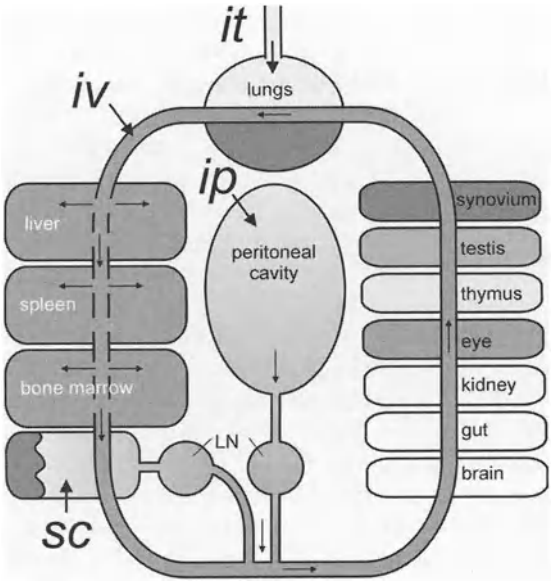

Fig. 1 Routes of administration of liposomes to reach macrophages. Intravenous (*iv*) injection of liposomes will reach all macrophages in those organs that are in direct, open contact with the bloodstream, such as liver, spleen, and bone marrow. Intraperitoneal injection (*ip*) will reach first the macrophages in the peritoneal cavity, but may ultimately also reach macrophages in the lymph nodes that drain the peritoneal cavity and may from there even reach the bloodstream. The latter will especially occur when large or repeated doses are given i.p. Subcutaneous injection of liposomes will reach local macrophages in the subcutaneous tissues and from there will be transported by lymphatics to lymph nodes (*LN*). Again, here liposomes may ultimately reach the bloodstream. Other routes of administration, such as local, intratracheal (*it*) instillation, will lead to selected access to macrophages, as seen in the lung. Intratracheally administered liposomes will readily reach the alveolar macrophages, but will not reach the macrophages in the lung interstitium. Macrophages in the organs depicted on the *right* can be reached only by local injection of liposomes, but access is often difficult and incomplete

tion of clodronate, the cell will be eliminated by apoptosis. This approach has been named the liposome-mediated macrophage suicide technique (Van Rooijen and Sanders 1994).

By comparison of the doses of clodronate liposomesliposome required for depletion of macrophages and the time interval between injection of liposomes and depletion, ample information on the in vivo accessibility of various macrophages has been obtained. The data are summarized below.

3.2 Intravenous Administration

Intravenously injected materials can reach macrophages in the liver (Kupffer cells), spleen and bone marrow (Fig. 1). Kupffer cells in the liver sinuses, as well as marginal zone macrophages and red pulp macrophages in the spleen have a

strategic position with respect to large molecular aggregates and particulate materials in the circulation. Liposomes have a nearly unhindered access to these macrophages as concluded from their fast and complete depletion within 1 day after intravenous injection of clodronate liposomes in mice and rats (Van Rooijen et al. 1990). Obviously, it is a little more difficult for intravenously injected liposomes to reach the marginal metallophilic macrophages in the outer periphery of the white pulp. Depletion of the white pulp macrophages in the periarteriolar lymphocyte sheaths (PALS) is incomplete, emphasizing the barrier formed by the reticulin fiber network and/or the densely packed lymphocytes in the white pulp.

The spleen is often chosen as a model system to study macrophages in vivo, since five different subpopulations of macrophages can be found that are all restricted to their own compartment. Moreover, these macrophages are separated from the main blood flow by different barriers. Large molecules such as serum albumins and 500-kDa dextran are retained within the marginal zone and red pulp. Only small polysaccharides and proteins are able to permeate the splenic white pulp. Evidence has been presented that there exists a splenic conduit network that regulates the transport of small molecules in the white pulp (Nolte 2002).

Additionally, macrophages in the bone marrow have access to intravenously injected clodronate liposomes. However, two consecutive injections with a time interval of 2 days were required to get a nearly complete depletion of macrophages from the bone marrow (Barbe et al. 1996).

Kupffer cells in the liver play a key role in the homeostatic function of the liver. They form the largest population of macrophages in the body, make up 30% of the hepatic nonparenchymal cell population, and have easy access to particulate materials in the circulation. Consequently, a large proportion of all intravenously administered particulate carriers used for drug targeting or gene transfer will be prematurely destroyed before they reach their targets. Therefore, transient blockade of phagocytosis by Kupffer cells might be an important factor to optimize in drug targeting, gene transfer, and xenogeneic cell grafting (van Rooijen et al. 1997). Also, transient suppression of the cytokine-mediated activity of Kupffer cells might have a beneficial effect on various disorders of the liver (Schumann et al. 2000).

3.3
Subcutaneous Administration

Subcutaneously injected clodronate liposomes are able to deplete macrophages in the draining lymph nodes of mice and rats (Fig. 1). Such liposomes, when, e.g., injected in the subcutaneous tissue of the footpads of mice, led to the depletion of subcapsular sinus lining macrophages and medulla macrophages in the draining popliteal lymph nodes (Delemarre et al. 1990). Macrophages in the paracortical fields and those in the follicles of the lymph nodes were not affected, emphasizing the existence of a barrier formed by reticular fibers and/or

densely packed lymphocytes in these lymph node compartments, comparable to that formed in the white pulp of the spleen.

After passing the popliteal lymph nodes, the lymph flow will be further filtered by consecutive draining lymph node stations such as the lumbar lymph nodes (in the mouse). Obviously, macrophages in these secondary lymph nodes were less efficiently depleted. Interestingly, only macrophages had been depleted in those lymph node compartments that directly drained the initial popliteal lymph nodes, indicating that different parts in the lymph nodes are corresponding each with their own draining area and have their own afferent lymph vessels. As a consequence, particles such as liposomes are not equally distributed over all macrophages in the lymph nodes.

3.4
Intraperitoneal Administration

Macrophages from the peritoneal cavity and the omentum of the rat can be depleted by two consecutive intraperitoneal injections with , given at an interval of 3 days (Biewenga et al. 1995). The peritoneal cavity is drained by the parathymic lymph nodes (in rats and mice). After passing these lymph nodes, the lymph flow reaches the blood circulation via the larger lymph vessels such as the ductus thoracicus. As a consequence, intraperitoneally injected clodronate liposomes are also able to deplete the macrophages of parathymic lymph nodes and, once they arrive in the blood circulation, they may deplete macrophages in liver and spleen (Fig. 1). Given the relatively large volume that can be administered in a single dose via the intraperitoneal route, the total number of macrophages that can be affected is even higher than that affected by intravenous injection.

3.5
Intratracheal Instillation and Intranasal Administration

Alveolar macrophages form a first line of defense against microorganisms entering the lung via the airways. In contrast to the interstitial macrophages that are separated from the alveolar space by an epithelial barrier, alveolar macrophages which are located in the alveolar space have direct access to liposomes administered via the airways, for instance by intratracheal instillation, intranasal administration, or by the application of aerosolized liposomes (Fig. 1). The direct access of clodronate liposomes to alveolar macrophages is demonstrated by the rapid elimination of these cells after intratracheal administration into mice and rats (Thepen et al. 1989), leaving the interstitial macrophage population unaffected. Alveolar macrophages make up about 80% of the total macrophage population in the lung. Given their presence in high numbers and the total mass of lung tissue, they form an important population of macrophages in the body.

3.6
Intraventricular Injection in the Central Nervous System

Stereotaxical injection of clodronate liposomes into the fourth ventricle of the central nervous system (CNS) of rats resulted in a complete depletion of perivascular and meningeal macrophages in the cerebellum, cerebrum, and spinal cord of these rats (Polfliet et al. 2001). These results also confirm that macrophages in the brain are accessible to liposomes if the latter are administered via appropriate routes.

3.7
Intra-articular Injection in the Synovial Cavity of Joints

Phagocytic synovial lining cells play a crucial role in the onset of experimental arthritis induced with immune complexes or collagen type II. A single intra-articular injection with clodronate liposomes caused the selective depletion of phagocytic synovial lining cells in mice and rats, demonstrating that this administration route allows easy access of the liposomes to the macrophages lining the synovial cavity. Recent experiments have confirmed that liposomes are also able to reach synovium lining macrophages in man (Barrera et al. 2000).

3.8
Local Injections: The Testis

Local injection of a suspension of liposomes can be performed in most organs. However, whether or not the liposomes will be able to diffuse from the injection site over the rest of the tissue will largely depend on the tissue structure. In the testis of rats, a loosely woven tissue structure allows the liposomes to reach most of the testicular macrophages, as demonstrated by the finding that at least 90% of the testicular macrophages can be depleted by clodronate liposomes (Bergh et al. 1993).

4
Specificity of Liposome-Mediated Delivery into Macrophages

During the past decade, several particulate drug-carrier devices have been developed. Among these, liposomes may be considered the most versatile and promising drug-delivery system (e.g., Gregoriadis 1995). The natural fate of liposomes, once they have been injected in vivo, is their uptake and degradation by macrophages. As a consequence, both liposome-encapsulated hydrophilic molecules and hydrophobic molecules that are associated with the phospholipid bilayers of the liposomes will be targeted into the macrophage, if no action is taken to prevent their phagocytosis. Although macrophages in general seem to prefer liposomes with an overall negative charge, e.g., achieved by incorporation of the anionic phospholipid phosphatidylserine in the bilayers, neutral and cat-

ionic liposomes are also rapidly taken up by macrophages. Several modifications of the original liposome formulations, such as the incorporation of amphipathic polyethylene glycol (PEG) conjugates in the liposomal bilayers have been proposed in order to reduce the recognition and uptake of liposomes by macrophages. Nevertheless, a large percentage of these so-called long-circulating liposomes will still be ingested by macrophages, emphasizing that macrophages form the logical target for liposomes (Litzinger et al. 1994). Liposomes of more than one micron in diameter will be internalized by non-phagocytic cells to a very low extent only. This explains why other cells such as lymphocytes and granulocytes are not depleted by clodronate liposomes. In summary, it may be concluded that liposomes form an ideal vehicle for the delivery of various compounds into macrophages.

Given the fact that macrophages will ingest all types of non-self macromolecules and particulate materials, it is difficult to achieve specific targeting to only one macrophage subset, e.g., in the spleen. In studies intended to reveal the conditions for monoclonal antibody-mediated specific targeting of enzyme molecules to marginal metallophilic macrophages in the spleen, we found that highly specific targeting of the enzyme molecules could be achieved only by using monomeric conjugates of the antibody and the enzyme. Larger conjugates lead to their uptake by all macrophage subsets in the spleen (van Rooijen et al. 1992).

5 Conclusions

Macrophages play key roles in many processes of the body. To stimulate or downregulate their functions in therapeutic ways using their extremely efficient phagocytosis capacity, one has to consider that they are often located at sites that are hard to reach. Local administration of drugs is therefore often the method of choice, whereby liposomes seem to be an ideal vehicle through their versatility and relative ease of handling, as well as their efficient uptake by macrophages.

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Antigen-Presenting Cells and Vaccine Design

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Abstract The innate immune system modulates antigen-specific adaptive immune responses in a qualitative and quantitative manner. In this chapter we propose that vaccine design would benefit from decoding this instructive function. We argue that the characterisation of cellular (particularly antigen-presenting cells) and soluble components of the innate system, and how they are interconnected, is an essential first step towards this goal.

Keywords Adaptive immune system, Adjuvants, Carbohydrate antigens, Marginal zone metallophilic macrophages, B cells, Innate immune system, T cells

Abbreviations

APC	Antigen-presenting cell
CR	Complement receptor
DC	Dendritic cell
Ig	Immunoglobulin
LPS	Lipopolysaccharide
MALT	Mucosal associated lymphoid tissue
LRR	Leucine-rich repeat
MØ	Macrophage
MR	Mannose receptor
MZ	Marginal zone
PAMP	Pathogen associated molecular pattern
Sn	Sialoadhesin
SR-A	Scavenger receptor class A
ss	Subcapsular sinus
TD	T-cell dependent
TI	T-cell independent
TLR	Toll-like receptor
TM	Transmembrane

1 Approaches to Vaccines

Historically, the aim of vaccination has been to prevent outbreaks of infectious diseases. Traditional vaccines were developed to mimic natural infections with live attenuated or inactivated bacteria or viruses. Live attenuated bacterial and viral vaccines include those for *Mycobacterium tuberculosis* (Bacille Calmette Guerin or BCG), *Salmonella typhi* Ty21a, vaccinia, polio, measles, mumps and rubella. Inactivated vaccines include those for *Vibrio cholerae*, *Bordetella pertussis*, influenza and rabies. Although their benefit-to-risk ratio is high, there is little or no knowledge of the protective antigens or immune responses responsible for immunity. Unpredictable adverse effects associated with a few conventional vaccines [eg. *B. pertussis* (Cody et al. 1981), rotavirus (Lynch et al. 2000) and respiratory syncytial virus (Kapikian et al. 1969)] are a major drawback of this empirical approach to vaccine development. Conventional vaccines with established high efficacy and safety would continue to contribute to vaccine programs, but current experimental vaccines in humans have been directed toward the use of well-defined antigens (subunit vaccines) to generate protective immunity. This rational approach has been made possible with our increased under-

standing of the molecular details of microbial pathogenesis, immune responses against microbial targets, and humoral and cell-mediated immune mechanisms. Subunit vaccines are either purified from bacteria or viruses or produced chemically or by recombinant gene technology. Subunit bacterial vaccines include formaldehyde-inactivated exotoxins from *Corynebacterium diphtheriae* (diphtheria toxoid) and *Clostridium tetani* (tetanus toxoid), and capsular polysaccharides from a number of encapsulated bacteria (e.g. *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*) (Levine et al. 1997).

The present aims for vaccination are no longer restricted to the prevention of infectious diseases, but also include therapeutic intervention of chronic viral infections, cancers, alloreactivity, autoimmunity and allergy. Contraceptive vaccines are also under development (Levine et al. 1997). The types of immune responses that vaccines are required to elicit or inhibit vary with the nature of the pathogens and targeted antigens or conditions. For most extracellular pathogens, antibody responses appear to be important, whereas for most intracellular pathogens, cell-mediated responses are responsible for protection. A combination of both humoral and cell-mediated responses might be required for elimination of some pathogens. Whilst inducing neutralising antibodies against toxins or surface molecules on pathogens has been the basis for almost every past and present successful vaccine, there are many important human pathogens (e.g. malaria and HIV) and tumours that require the elicitation of strong cell-mediated immune responses. There is also a need for vaccine formulations able to induce immunological tolerance or modulate T-helper cell responses for conditions such as autoimmunity and allergy (Liu 1997).

Identification of suitable B- and T-cell epitopes (class II and class I restricted) that are targeted by the acquired immune system is necessary but insufficient for the development of effective vaccines. It has to be complemented by investigation of delivery systems and formulations (collectively referred to as adjuvants) that would compensate for the inherent lack of immunogenicity of subunit vaccines. Most adjuvants appear to act through their interactions with the innate immune system (Moingeon et al. 2001). The current view that the innate immune system not only provides short-term protection but also instructs the adaptive immune system to generate the most appropriate B- and T-cell responses following its encounter with pathogens is supported by recent evidence (Medzhitov and Janeway 2002). Thus, understanding the links between the innate and the adaptive immune systems is critically important to vaccine design.

2 Immune Responses to Antigens

Immune responses against microbes and antigens are initiated in secondary lymphoid organs, spleen, lymph nodes and mucosal-associated lymphoid tissue (MALT). In order to elicit an immune response, antigens entering at peripheral sites such as the skin, blood and gastrointestinal tract need to reach these secondary lymphoid organs where antigen-specific naïve B and T lymphocytes cir-

culate. Not every antigen can elicit an immune response. Immunogenicity appears to vary with antigen form [soluble, cell-associated (Batista et al. 2001), or particulate (Ramachandra et al. 1999)], amount or load (Zinkernagel 2000), composition (foreignness) (Allison and Fearon 2000; Apostolopoulos et al. 2000) and location within the body (mucosal or systemic sites). It also depends on their propensity to activate complement (Carroll 1998) or engage other serum factors and natural antibodies (Sakamoto et al. 2001; Shimizu et al. 2001), and perhaps more importantly the context (Shi and Rock 2002) (homeostasis or inflammation) in which they are detected by the innate immune system.

Lack of immunogenicity to self-antigens is of obvious benefit. Tolerance to self-antigens is mostly due to the stringent process of negative selection of B and T lymphocytes in bone marrow and thymus, respectively. This central tolerance mechanism ensures the deletion of most autoreactive B and T cells. For those B and T cells that escape negative selection in the primary lymphoid organs, their full activation by self-antigens is prevented by peripheral tolerance mechanisms by which they could be killed, anergised or negatively regulated. Activation of naïve antigen-specific B and T cells to proliferate and differentiate into effector and memory cells requires antigen-presenting cells (APC) to capture, process, transport and present antigens, and provide co-stimulatory signals and cytokines.

Antigens reaching lymphoid organs are either native (cell-associated or soluble) or processed by APC. Native antigens transported with bound complement and/or antibodies or by specialised APC (Wykes et al. 1998; Berney et al. 1999) are recognised by antigen-specific B cells and are deposited on the surface of follicular dendritic cells (FDC) (Tew et al. 2001). In the case of T cell-dependent antigens (TD), B cells will acquire T-cell help and form germinal centres where immunoglobulin (Ig) class switching, affinity maturation and the production of memory cells occur. This is the case for most protein antigens and some glycolipids that can be presented to T cells by MHC class II or CD1 molecules, respectively. Humoral immune responses can also be elicited in the absence of classical B–T cognate interactions. These thymus independent (TI) responses are essential in the initial protection against pathogens and are characterised by the lack of Ig class switching and memory B cells. TI type 1 antigens [such as lipopolysaccharide (LPS)] induce a polyclonal response by activating B cells in an antigen-independent manner. TI type 2 antigens are normally polymers [carbohydrates or proteins assembled as multimers including components of viral capsules (Fehr et al. 1998)] that are able to cross-link B-cell receptors and induce mainly IgM and IgG3 production in mice and IgM and IgG2 in humans. At large antigen doses, all lymphoid organs are able to elicit TI responses. However at more realistic antigen doses, only the spleen can accumulate enough antigens to trigger TI responses (Ochsenbein et al. 2000). Therefore, the spleen has been considered as essential for the generation of TI responses.

3
Professional Antigen-Presenting Cells and Immunity: The Balance Between Clearance and Presentation

It is becoming clear that APC responses to microbes and their unique products are critical to the initiation of immune responses against pathogens. Macrophages (MØ) and dendritic cells (DC) appear to be the most important APC. Both cell types are widely distributed and located suitably throughout the body to encounter pathogens and microbial products. MØ are endowed with a number of cell surface receptors involved in opsonic and nonopsonic endocytosis and phagocytosis and upon activation produce soluble mediators that could initiate and regulate inflammatory responses. Despite having similar sets of cell surface receptors and function in antigen processing, MØ and DC have different roles in the induction and regulation of immunity, with DC being uniquely suited to activate naïve T cells in secondary lymphoid organs (Steinman et al. 2000). DC act as messengers, and instruct T cells to respond to stimuli they have received in peripheral tissues (Lanzavecchia and Sallusto 2001). The instructions would be conveyed in several ways: by the peptides presented on MHC-I and -II molecules, by the co-stimulatory and adhesion molecules displayed at the cell surface, and by the cytokines produced by the APC. The nature of the antigen and the inflammatory response elicited in the periphery would determine the subsequent B- and T-cell responses (Hawiger et al. 2001). Vaccine development will thus benefit from the identification of the mechanisms regulating APC function in situ.

Characterisation of the resident and recruited APC populations and their behaviour upon stimulation would enable the design of vaccines that will target antigens and APC to the appropriate secondary lymphoid organs and generate suitably activated T-and B-cell populations. This approach is of great interest in the case of prophylactic vaccines to be used in healthy individuals, since it will focus on delivery methods and formulations that can be produced in large scale. Therapeutic vaccines aiming at modifying an ongoing immunological process (such as allergy and chronic infection) require an understanding of the mechanisms that govern the conditions of non- or hyper-responsiveness of local APC populations, the regulation of activated T cells and the targeting systems that would act locally.

Monocytes migrate constitutively to tissues where they normally differentiate into MØ and in some cases DC (Randolph et al. 1998). MØ are heterogeneous in shape and function, depending on their local microenvironment, and can be distinguished by differences in the level of expression of certain cell surface molecules (Gordon 1999). During steady-state conditions, MØ remain in the tissue where they play an important role in normal homeostasis. Their unique endocytic and phagocytic capacity place MØ at the centre of the inflammatory process because they have the advantage of sampling the environment at the first instance (Parnaik et al. 2000; Fadok and Chimini 2001). As such, they could effectively control the availability of antigens encountered by DC. This could be

relevant in the case of autoimmunity, since MØ reduce the amount of self-antigens that might be available to break tolerance. They also perform a more active role in the prevention of autoimmunity through the production of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β upon apoptotic cell uptake (Fadok and Chimini 2001). These inhibitory properties have been exploited by some intracellular microbes that have developed mechanisms to gain access to the MØ intracellular environment (Ernst 1998) and are most apparent in alveolar MØ, which have also been shown to inhibit DC function (Goerdt et al. 1999; Tzachanis et al. 2002). To perform homeostatic functions, tissue MØ express endocytic receptors such as the mannose receptor (MR) and scavenger receptors, but not MHC-II and co-stimulatory molecules. This phenotype might be controlled through the interaction of the widely expressed OX2 (CD200) with its ligand (OX2L) on MØ (Hoek et al. 2000; Wright et al. 2000), or through the action of Ig-like inhibitory receptors (Dietrich et al. 2000). Finally, MØ are effector cells of the innate immune system. They are capable of destroying microbes and tumour cells and could cause tissue damage through the release or impaired clearance of inflammatory mediators, lysosomal hydrolases and reactive oxygen and nitrogen radicals. This capacity will be enhanced or modulated once activated T cells reach the tissues. In particular, T-helper (Th)1 T cells will enhance cytotoxic MØ mechanisms through the production of interferon (IFN)-γ. Th2-derived cytokines have a more subtle effect and seem to generate MØ with an alternatively activated or suppressor phenotype characterised by enhanced expression of endocytic receptors, reduced T-cell activation capacity, arginase production and synthesis of alternative macrophage-associated chemokine (AMAC)-1 (Stein et al. 1992; Goerdt et al. 1999; Tzachanis et al. 2002).

Under steady-state conditions, DC migrate through lymphatics to secondary lymphoid organs (Huang et al. 2000). This migratory DC population found in the absence of inflammation has not been well characterised, but might be involved in the maintenance of tolerance. They could transport self-antigens and present them to naïve T cells either directly, or through antigen transfer to resident DC (Steinman et al. 2000). When an inflammatory response is elicited through injury, infection or active immunisation, recruited monocytes move into the tissues and differentiate into activated MØ with increased endocytic and secretory activities. Under these conditions, enhanced cell trafficking to draining lymphatic tissue ensures antigen delivery and presentation. Though DC have been considered the likely mediators of antigen transport, several reports have identified recruited MØ as the major cells involved in antigen capture in situ. In a fluorescent microsphere-induced inflammation model, recruited monocytes with a MØ-like phenotype could be seen internalising these particles in the periphery. Antigen-bearing cells in lymphoid organs were then characterised as DC (Randolph et al. 1999). In the case of the adjuvant MF59, recruited cells at the site of injection that internalised the adjuvant were characterised as MØ ($F4/80^+$, $CD11b^+$, $CD11c^-$). When draining lymph nodes were analysed, MF59-loaded apoptotic MØ were observed at the subcapsular sinus, and a sec-

ond MF59-containing cell population was detected in the T-cell area. These cells presented adjuvant-containing apoptotic bodies, indicating that antigen transfer between both cell types could have taken place (Dupuis et al. 2001).

Microbial-derived products exert a major influence in the phenotype of APC. Much research has focused in defining their capacity to modulate DC phenotype and determine the differentiation pathway (Th1 vs Th2) of Th cells upon activation (Lanzavecchia and Sallusto 2001). The use of large doses (e.g. 25 μg LPS/mouse) of antigens in most of these studies has made the assessment of the role of MØ in modulating these processes difficult (Pulendran et al. 2001). It is likely that MØ clearance/signalling systems could be overwhelmed by large doses of antigens. Under more physiological conditions, MØ could influence the extent of the inflammatory response by expressing inhibitory factors such as IL-10 following stimulation (Sutterwala et al. 1998; Gerber and Mosser 2001). IL-10 could act as a de-activator and induce uncoupling of the cytokine receptors required by DC for migration to lymphoid organs (D'Amico et al. 2000). In a model of soluble *Toxoplasma gondii* tachyzoite antigen (STAg)-induced DC migration and IL-12 production, lipoxin produced by splenic MØ induces a refractory phase in the responsiveness of DC to this stimulus (Aliberti et al. 2002).

4 Role of MØ in Secondary Lymphoid Organs in the Control of Immune Responses

There are distinct populations of MØ in secondary lymphoid organs. Blood-borne antigens are encountered by the spleen, which, as mentioned earlier, is required for TI-2 responses both in humans and mice (Amlot et al. 1985). This functional requirement in both species does not translate into structural similarity. Even though in both cases the marginal zone (MZ) is populated by B cells with an activated phenotype ($CR2^+$, IgD^-, IgM^+, $CD27^+$) distinct from the follicular recirculating pool (IgD^+, IgM^+) (Zandvoort et al. 2001), human spleen lacks a distinct marginal sinus, but contains an additional structure referred to as the perifollicular zone which surrounds B-cell follicles. However, a population of sialoadhesin $(Sn)^+$ MØ, a characteristic feature of murine MZ, has been located at these perifollicular areas (Steiniger et al. 1997). In murine spleens there are five MØ subtypes (Fig. 1). Red pulp MØ ($F4/80^+$, $macrosialin^+$, MR^+) do not seem to play a direct role in the regulation of immune responses except for antigen clearance and probably for their release of soluble mediators following activation. White pulp (WP) MØ are poorly characterised. They lack F4/80 expression but can be visualised by their expression of macrosialin. White pulp MØ, identified with the MOMA-2 monoclonal antibody, might act in the enhancement of immune responses through the regulated synthesis of C3 after immunisation, which could induce local opsonisation of antigens (Fischer et al. 1998). During the germinal centre reaction, tingible body macrophages ($macrosialin^+$, MR cysteine-rich domain $ligands^+$) (Martinez-Pomares et al. 1996) can be ob-

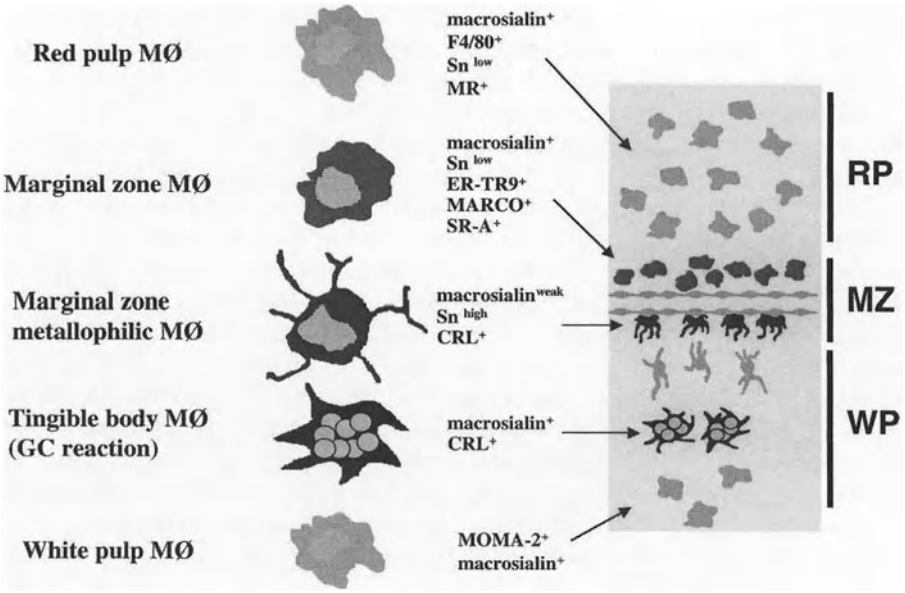

Fig. 1 In addition to their particular anatomical localisation, splenic MØ can be classified based on their differential pattern of marker expression. While red pulp MØ exclusively express the EGF-TM7 family member F4/80 and MR, they share expression of macrosialin with other MØ populations. Expression of the scavenger receptors MARCO and scavenger receptors class A, and the DC-SIGN murine homologue mSIGNR1 (recognised by the ER-TR9 antibody) is restricted to marginal zone MØ. Marginal zone metallophilic MØ are uniquely endowed with high expression of the member of the Siglec family, Sn, and the ability to interact with the CysR domain of the MR. Evenly distributed within lymphoid areas, a population of cells expressing macrosialin can be observed. During the germinal centre reaction, macrosialin expressing MØ that can bind the CysR domain of the MR (tingible body MØ) can be observed in the dark zone. These cells are probably involved in clearance of apoptotic cells

served in the dark zone. These cells are thought to be involved in the uptake of apoptotic B cells.

In murine spleens, the MZ (Kraal 1992) is a unique structure surrounding the marginal sinus where two distinct MØ populations are situated. The marginal zone metallophilic (MZM) MØ, which surround the white pulp but are more abundant around follicular areas, are strategically placed to be exposed to antigen and to control cell trafficking. These cells express large quantities of Sn, a member of the Siglec superfamily of proteins involved in cell adhesion. MZM-MØ migrate into the lymphoid area under some circumstances and could be involved in the transport of antigens to the follicles (Groeneveld et al. 1986; Yu et al. 2002). They express sulphated glycoforms of Sn and CD45 that act as counter-receptors for the cysteine-rich (CysR) domain of the MR (Martinez-Pomares et al. 1999). This interaction suggests that MR^+ cells or soluble MR (sMR) can be targeted to these cells. Interaction between membrane lymphotoxin expressed by B cells with its receptor on stromal cells is required for the induction of the sulphotransferases responsible for the generation of the sulphated glyco-

proteins that mediate CysR-domain binding (Martinez-Pomares et al. 1999; Yu et al. 2002). One day following LPS immunisation, MZMMØ are detected in the follicles. They maintain the ability to bind the CysR domain of the MR, and express MOMA-1 and Sn (Groeneveld et al. 1986; Yu et al. 2002). However, following immunisation with sheep red blood cells, the expression of the murine homologue of the human germinal centre DC marker decysin in MZMMØ was induced. Decysin$^+$ cells expressing ligands for the CysR domain were detected at 48 h in follicles (Mueller et al. 2001). These results suggest that these cells possess the flexibility to modulate their phenotype according to the type of antigen injected.

MZMMØ are of special interest in adjuvant and vaccine development for three main reasons. They are targeted by immunostimulating complexes or ISCOMs after intravenous immunisation (Claassen et al. 1995); they are required for TD responses to particulate bacterial antigens (Buiting et al. 1996); and they are major producers of IFN-α/β after viral infection (Eloranta and Alm 1999). In contrast to DC, these cells are macrophage colony-stimulating factor (M-CSF)-dependent, can be eliminated using liposomes containing clodronate (van Rooijen et al. 1989), and lack MHCII expression in situ (L. Martinez-Pomares, unpublished).

At the other side of the marginal sinus resides a population of large MØ (MZMØ) that express clearance receptors such as scavenger receptors class A (SR-A) (Hughes et al. 1994), macrophage receptor with collagenous domain (MARCO) (Elomaa et al. 1995) and an uncharacterised neutral polysaccharide receptor detected by the antibody ER-TR9 (van Vliet et al. 1985). Though it has been suggested that these cells could be involved in the generation of TI responses, depletion experiments using ER-TR9 (Kraal et al. 1989) seem to indicate that this is not the case. Indeed they might play a down-modulating role by reducing antigen load (Van den Eerwegh et al. 1992). A role in processing particulate antigens and transfer to MZ B cells cannot be ruled out. The induction of the initial TI, neutralising IgM response to vesicular stomatitis virus, poliomyelitis virus and recombinant vaccinia virus is dependent on efficient antigen trapping by CR3- and CR4-expressing MØ of the marginal zone (Ochsenbein and Zinkernagel 2000).

Material collected by lymphatics drains into lymph nodes (LN). The first cells encountered by this mixture of plasma components and cells (migratory DC among them) are the subcapsular sinus (ss) MØ. This population of M-CSF-dependent MØ express Sn and MOMA-1 and can be depleted using clodronate-loaded liposomes injected subcutaneously (van Rooijen and Sanders 1994). Their presence depends on a constant flow of lymph, since elimination of lymphatic vessels leads to their disappearance from the ss and migration into T-cell areas (Mebius et al. 1991). As in the case of MZMMØ, ssMØ express a sulphated glycoform of Sn that can be recognised by the CysR domain of the MR (Martinez-Pomares et al. 1996). After immunisation, a migratory cell population with the same phenotype but expressing CD11c and MHC-II can be found in the follicles. These cells are functionally DC and present antigen to naïve T and

B cells in vivo (Martinez-Pomares et al. 1996; Berney et al. 1999). Their origin is not clear; they could derive from the ssMØ or represent migratory cells moving from the periphery. Another major MØ populations present in LN are the medullary MØ that surround lymphatic and vascular endothelia at the medullary region. They seem to be involved in clearance and accordingly express similar markers to those found on MZMØ such as SR-A and MARCO (Elomaa et al. 1995) in addition to F4/80, MR and Sn (Linehan et al. 1999). MZMMØ and ssMØ could correspond to the antigen-transporting cells that transport immune complexes during the course of an immune response as previously described by Szakal et al. (1983). Cells expressing the MØ calcium-type lectin, found on dermal MØ but not Langerhans cells, could be transiently found in the T-cell areas of lymph nodes during the sensitisation phase of contact hypersensitivity and seemed to contribute to the efficiency of sensitisation even though they were negative for the antigen. This work opens the possibility of a modulator role for migratory MØ during the process of T-cell activation (Sato et al. 1998). Beside macrophages, immature and mature DC also reside in LN. They are able to internalise and process antigens. It has been proposed that a resident DC population in lymphoid organs could be implicated in tolerance induction (Steinman et al. 2000).

5
Adjuvants as Immunomodulators and Delivery Systems

Adjuvants are substances that enhance immune responses in general or specific to a particular antigen. Original adjuvant formulations consisting of breadcrumbs, agar, saponin, starch oil and lecithin were found to increase the levels of diphtheria or tetanus antitoxin (Ramon 1925). This empirical approach has identified a wide spectrum of substances as candidate adjuvants. These include bacterial and plant products, surface-active agents such as saponins, synthetic and biopolymers, vitamins, hormones, and aluminium and calcium salts. Their modes of action in the immune system are generally not known. To date, Alum is the only adjuvant approved for human vaccines (Edelman 2002). This is probably because it is extremely safe, rather than its effectiveness in enhancing all immune responses. Alum can only enhance antibody responses in some vaccines, and it is totally ineffective in stimulating cell-mediated immune responses. There is thus a great need for understanding the modes of action of experimental adjuvants or adjuvant formulations in order to improve current vaccines and to design novel ones. The aims for modern adjuvants or adjuvant formulations would be to boost the immunogenicity of subunit vaccines at mucosal sites or generate antigen-specific B and T cells by activating selectively Th-cell subsets (Th1 or Th2). Adjuvants could act in several potential ways. They could introduce or maintain antigen in the appropriate in vivo microenvironment. They could recruit and activate APC and lymphocytes by providing signals associated with infections, which would induce inflammation and increase Ag delivery to draining lymphoid tissues. Finally, they could deliver T-cell epitopes to

MHC class-I and -II molecules for CD8 CTL and CD4 Th cells, respectively [see O'Hagan et al. (2001) for review].

6
Endocytic Nonopsonic Receptors on Antigen-Presenting Cells as Molecular Targets for Antigen Delivery

Endocytic receptors on DC and MØ could be considered as targets for antigen delivery to MHC-II compartments and subsequent enhanced antigen presentation to T cells. In some instances, targeting to MHC-I compartment through the exogenous pathway can also take place. Since many of these receptors recognise endogenous molecules (Platt and Gordon 1998; Linehan et al. 2001; Lee et al. 2002), a balance between clearance and presentation could be established through their regulated differential expression on MØ and DC. Additionally, the requirement for inflammatory stimuli would ensure that peripheral tolerance is maintained.

6.1
The Mannose Receptor and the Scavenger Receptor Class A

In the mouse, MR and SR-A are absent from conventional DC populations in the periphery and lymphoid organs (Hughes et al. 1995; Linehan et al. 1999). This would be in accordance with their role as clearance systems for a large number of self-molecules (Table 1). Nonetheless, their presence in bone marrow-derived DC (both MR and SR-A, unpublished observations) and in cultured human DC (only shown for the MR) suggests that they might be up-regulated during DC differentiation or recruitment in vivo. Indeed, an uncharacterised population of MHC-II$^+$, MR$^+$ cells has been described in LN (Linehan et al. 1999), and MR expression has also been observed in situ in human inflamed skin (Wollenberg et al. 2002). SR-A is highly expressed by MZMØ (Hughes et al. 1995). In both cases enhanced presentation has been observed after mannosylation (Sallusto 1995; Agnes et al. 1997) or maleylation (Bansal et al. 1999) of proteins to generate MR and SR ligands respectively. MR can mediate the uptake and targeting of glycolipids for presentation by CD1b in human monocyte-derived DC (Prigozy et al. 1997). These properties were exploited in the induction of protective anti-tumoral responses by immunisation with the tumour-associated antigen MUC-1 conjugated to mannan, a ligand for the MR (Apostolopoulos et al. 1995, 2000). However, MR-mediated enhanced presentation needs to be reassessed in view of the results of several recent studies: (1) LPS-induced IL-12 production in cultured human DC can be inhibited by mannosylated lipoarabinomannans (Nigou et al. 2001); (2) The maturation state of DC is not affected by the involvement of MR in the uptake of secretory IgA (Heystek et al. 2002); and (3) MR can block the intracellular sorting and presentation of MUC-1 (Hiltbold et al. 2000). Therefore, in the absence of additional stimuli, MR ligation might not be able to enhance immunogenicity of antigens or activate DC.

The presence of MR CysR domain ligands in selected myeloid populations in secondary lymphoid organs (Martinez-Pomares et al. 1996; Berney et al. 1999; Martinez-Pomares et al. 1999; Yu et al. 2002), highlights the dual role of the MR in homeostasis and immunity. A soluble form of the MR (Martinez-Pomares et al. 1998) has been suggested as the counter-receptor for these molecules.

6.2 DEC-205 and DC-SIGN

DEC-205 (Table 1) is an endocytic receptor mostly restricted to DC populations and thymic epithelium. Even though its ligands have not been identified, using polyclonal and monoclonal antibodies it has been shown that its cytoplasmic tail is highly efficient in targeting to late endosomes and to the MHC-II compartment (Jiang et al. 1995; Mahnke et al. 2000). Recently a chimeric anti-DEC-205 monoclonal antibody bearing a hen egg lysozyme T cell epitope was shown to stimulate antigen-specific T cells in vivo. The outcome of this stimulation is unresponsiveness if this reagent was injected in the absence of adjuvant or activation and if an anti-CD40 monoclonal antibody was used (Hawiger et al. 2001). Nevertheless, these results should be carefully assessed since DEC-205 can be expressed by MØ under inflammatory conditions (Wijffels et al. 1991).

Table 1 Selected antigen-presenting cell receptors relevantin vaccine design

Receptor	Ligand	Structure	Comments
Mannose receptor	SO_4-3-Gal-SO_4-(3/4)-GalNAc-mannose, fucose *N*-acetyl-glucosamine-	CysR domain-FNII-CRD(x8)-TM-CT (endocytosis)	
Scavenger receptor Class A	Selected polyanionic compounds	Collagenous domain Type1 only SRCR domain	
DEC-205	Unknown	CysR domain-FNII-CRD (x10)-TM-CT (endocytosis)	
DC-SIGN	Mannose *N*-acetyl-glucosamine	C-type lectin-(internalisation motif)	
TLR-2	Peptidoglycan Lipoprotein Lipoarabinomannan	LRR	Signals as heterodimer in combination with TLR1 and TLR6
	Selected lipopolysaccharides	Cytoplasmic domain related to IL-1R (TIR domain)	
TLR-3	dsRNA	LRR and TIR	
TLR-4	Lipopolysaccharide Lipoteichoic acids	LRR and TIR	Requires MD2 and CD14
TLR-5	Flagellin	LRR and TIR	
TLR-7/TLR-8	Small anti-viral compounds	LRR and TIR	
TLR-9	Unmethylated CpG	LRR and TIR	
β-Glucan R	β-Glucan	C-type lectin-TM-CT (ITAM)	β-Glucan-independent binding to T cells

Another DC restricted receptor, DC-SIGN, a C-type lectin involved in T cell adhesion (through its interaction with ICAM-3), HIV-1-binding and transendothelial migration (through its interaction with ICAM-2) has been implicated in antigen internalisation and presentation to T cells (Engering et al. 2002). The requirement for antigen targeting to an endocytic receptor on DC might not be absolute since some studies indicate that targeting to other surface molecules on DC is enough to enhance presentation (Serre et al. 1998).

7 Signalling Receptors on Antigen-Presenting Cells as Sensors of Foreignness

Toll-like receptors (TLR) were thought to be genuine detectors of pathogen-associated molecular patterns (PAMP) (Medzhitov and Janeway 2000). This assessment is being challenged by the discovery of TLR-mediated signalling in responses to endogenous ligands that could be released from damaged cells (Leadbetter et al. 2002; Vabulas et al. 2002). In humans there are 10 members of this family and PAMP on specific microbes have been identified for most of these TLR (Table 1) (Akira et al. 2001; Medzhitov 2001; Hemmi et al. 2002; Kaisho and Akira 2002). Although direct binding of PAMP has not yet been demonstrated for any of the TLR, their signalling capability in response to PAMP is unequivocal. The homology of their intracellular region with the cytoplasmic tail of the IL-1R suggested that these molecules could signal through the same pathway, which involves an adaptor protein, My88D. Only TLR-4 has been shown to induce some responses through a MyD88-independent pathway mediated by another adaptor protein referred to as TIRAP or MAL (Horng et al. 2001). MyD88-deficient mice have a bias towards a Th2 response with enhanced basal levels of IgE and normal responses to ovalbumin (OVA) co-injected with Alum (a Th2-inducer) but not with complete Freund's adjuvant (CFA) (Th1-inducer). These results indicate that TLR signalling could favour the induction of a Th1 response (Schnare et al. 2001). TLR are differentially expressed in different DC populations, suggesting that there is specialisation of APC to respond to specific microbes (Kadowaki et al. 2001).

Finally dectin-1, widely expressed by myeloid cells including DC and MØ, has recently been shown to be a major β-glucan receptor on MØ (Brown and Gordon 2001; Brown et al. 2002). Since β-glucans from fungi and yeasts have a variety of immunostimulatory effects, this receptor could potentially mediate most if not all of these activities. It contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail and ligand recognition is expected to activate signalling pathways of DC and MØ. In addition a role for dectin-1 as an enhancer of innate immune responses, its phagocytic activity could also lead to enhanced antigen uptake and presentation.

8
Current Challenges of Vaccine Development

Mucosal delivery of antigens is one of the most desirable routes of immunisation because it is easier to administer, has reduced toxicity and provides immunity at the sites where many pathogens establish infection. However, oral immunisation using vaccines containing pure antigens or killed/inactivated organisms has not been successful in generating protective immunity. The difficulties have been the instability of antigens in the harsh acidic and proteolytic environment of the stomach and the limited access of antigens and APC stimuli to the mucin-coated epithelial cell layer and the underlying MALT. Some promising delivery systems and potent adjuvants for oral and intranasal immunisation include genetically modified non-toxic bacterial toxins [see (O'Hagan et al. 2001) and references therein], and microparticles with entrapped protein and DNA-encoded antigens (O'Hagan et al. 2001). The mechanisms for their effectiveness have not been defined, but it is most likely due to the fact that they can enhance antigen delivery to DC and MØ through pinocytosis and/or via their interactions with specific endocytic/phagocytic receptors, and activate these APC fully by the presence of PAMP. How these signals are integrated to achieve protective immunity is being investigated at the molecular and cellular levels.

Whilst almost all vaccines are targeted to elicit adaptive immune responses toward protein antigens, there is also a need to examine non-protein components as vaccine and adjuvant candidates. Carbohydrate antigens have an important role in vaccine development because immune responses against them could prevent successful blood transfusion, organ transplantation, tumour formation and microbial infections. In addition, some microbial carbohydrates, including alginate from *Pseudomonas aeruginosa*, capsular polysaccharides from *Klebsiella pneumoniae* and *Streptococcus pneumoniae* and β-glucans from yeasts and other fungi, have immunomodulatory effects on the immune system, and as such could also be used as adjuvants to improve the immunogenicity of vaccines against infectious diseases and cancer. Their modes of action have not been clarified, but induction of macrophage functions and cytokine production (e.g. TNF-α, IL-1) would likely be their effects (Yokochi et al. 1980; Otterlei et al. 1991; Otterlei et al. 1993; Choy et al. 1996; Ho et al. 2000; Tokunaka et al. 2000; Um et al. 2000; Suzuki et al. 2001).

On the surface of most human bacterial pathogens, carbohydrate antigens are in the form of capsular polysaccharides (CPSs), lipopolysaccharides/lipooligosaccharides, teichoic acids, and lipoteichoic acid. Antibody responses to CPSs are the basis for protection against most infections due to extracellular bacteria; but the efficacy of CPS-based vaccines varies with the antibody isotype and its complement-fixing ability. Antibody responses to CPS vary with age: adults and children over 2 years are generally responsive if CPS is immunogenic, but children under 18 months (who are the most vulnerable to bacterial infections) do not respond to CPS. To achieve T-cell help in order for B cells to produce antibodies against carbohydrates, investigators have coupled CPS chemi-

cally to protein carriers in an attempt improve their immunogenicity in infants. CPS-protein conjugate vaccines for type b *Haemophilus influenzae* and serogroup C *Neisseria meningitidis* have been extremely effective in reducing disease incidence to record low levels in countries where mass vaccination programs have been implemented.

Intranasal immunisation of a pneumococcal glycoconjugate with mucosal adjuvants such as *Escherichia coli* heat-labile enterotoxin mutants has recently been shown to induce a significant increase in polysaccharide-specific and protein carrier-specific antibody responses in all IgG subclasses (Jakobsen et al. 2001). Phenotypic characterisation and determining the functions of the DCs and MØs in the nasal-associated lymphoid tissues (NALT) should enable the design of effective adjuvants and vaccines for this practical route of immunisation against respiratory pathogens. Although the successes of conjugate vaccines have encouraged further development of similar vaccines against other bacterial pathogens, the underlying mechanisms of immune responses against carbohydrate antigens remain poorly defined. MØs and DCs have been shown to contribute to antibody responses to TI antigens by responding to PAMPs and activating TLR-mediated pathways of cytokine induction. The secreted cytokines, including IL-1, IL-6, IL-12 and GM-CSF, provide the necessary second signals to B cells that are activated by multivalent mIg cross-linking polysaccharides (Vos et al. 2000). It is, however, not clear how carbohydrate antigens are transported to secondary lymphoid tissues where antigen-specific B cells are located. Further investigations on DCs and MØs would also be required to understand the immunomodulatory properties of some polysaccharides. The challenge is to understand how these types of antigens interact with the innate immune system, and how recognition and potential signalling by receptors on DCs and MØs affect immunogenicity or adjuvanticity. Insights into these mechanisms would enable the development of vaccines designed to modulate immune responses against carbohydrate antigens or other similar TI antigens in normal and immunocompromised individuals. It may also be possible to identify novel carbohydrate-based adjuvants that can achieve a fine balance between initiation of immune responses without a potentially damaging inflammatory process, because almost all CPS-based vaccines are well-tolerated in humans.

In summary, DCs and MØs play critical roles in determining antigen tolerance and immunogenicity and the generation of appropriate adaptive immune responses. Future vaccine formulations would thus not only include selected B- and T-cell epitopes, but also delivery systems and adjuvant components that specially modulate the migration, antigen presentation and cell activation of specific DC and MØ types at targeted immune sites. The main challenges in vaccine development are to identify adjuvants to generate cell-mediated immune responses for tumours and chronic viral infections, and to enhance mucosal and systemic immune responses via the oral and intranasal routes. Detailed analyses of the immunobiology of DCs and MØs would contribute greatly to the designing of vaccines to meet these challenges.

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Macrophage-Specific Gene Targeting In Vivo

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Abstract The study of macrophage biology has been hindered by our inability to efficiently direct transgene expression to cells of the mononuclear phagocyte lineage in vivo. Recent progress in understanding the transcriptional regulation of several genes expressed predominantly in macrophages has led to improved vectors for macrophage gene targeting. Vectors that can reproducibly direct high-level, macrophage-specific transgene expression may find application in gene therapy protocols that seek to correct genetic defects of metabolism such as lysosomal storage disorders. Efficient targeting of gene expression to macrophages and dendritic cells may allow us to modulate immune responses and to develop more efficient genetic vaccines.

Keywords Phagocytes, Transgenes, Vaccines

1 Background

1.1 Introduction to the Cells of the Mononuclear Phagocyte Lineage

All blood cells develop from a series of haematopoietic progenitor cells, which ultimately originate from haematopoietic stem cells. Blood monocytes develop from monoblasts within the adult bone marrow and are released into the circulation. Monocytes are recruited via constitutive and inflammatory mechanisms to virtually all the tissues of the body where they terminally differentiate into the various cell types of the mononuclear phagocyte lineage. Mononuclear phagocytes in adult tissues include Kupffer cells in the liver, microglia in the brain, Langerhans cells in the skin and mucosa and tissue-resident macrophages in tissues such as the lamina propria detectable with specific monoclonal antibodies including F4/80 and FA/11, which recognise the gene products of the Emr1 and CD68 genes respectively. These tissue resident macrophages play important roles in tissue homeostasis and are key players in innate and adaptive immune responses. Other cell types of the mononuclear phagocyte family include osteoclasts which play an essential role in bone morphogenesis, bone remodelling and bone homeostasis. Monocytes can also differentiate into myeloid dendritic cells (DCs), which capture and process antigens in the tissues before presenting antigenic peptides to naïve T cells in the context of MHC class II within lymph nodes. While protocols for dendritic-cell differentiation from human monocytes in culture systems are well established, the exact linage relationships between macrophages and DCs in vivo are less well defined.

With the advent of new genomic technologies such as gene arrays, we have access to extensive databases that list the most highly expressed genes in macrophages, myeloid DCs, macrophages treated with oxidised low-density lipoprotein (LDL) and DCs challenged with different pathogens [to list only four recent papers: Hashimoto et al. (1999, 2000); Shiffman et al. 2000; Huang et al. 2001)]. One striking feature of this type of expression analysis is how few genes in these collections are solely restricted to mononuclear phagocytes. Among the "macrophage-restricted" genes of known function, we can count a number of pattern-recognition receptors including the scavenger receptors SR-A and MARCO as well as the macrophage mannose receptor (MMR) (McKnight and Gordon 1998). Other macrophage-restricted genes include the murine Emr1 gene, which encodes the antigen detected by the F4/80 monoclonal antibody (McKnight et al. 1996). Other members of this EGF TM7 family (Stacey et al. 2000) include the recently described membrane protein FIRE (Emr4), expressed by macrophages and DCs (Caminschi et al. 2001); Emr2, expressed by neutrophils and activated macrophages (Lin et al. 2000) and Emr3, expressed by neutrophils, monocytes and macrophages (Stacey et al. 2001). Other cell surface receptors that are restricted to mononuclear phagocytes include the M-CSF receptor, which is the product of the *c-fms* proto-oncogene and Siglec-1 a membrane lec-

tin expressed by a subset of macrophages that recognises carbohydrate ligands containing sialic acid (Munday et al. 1999). Recently we have cloned and characterised a number of macrophage-restricted genes including two cell surface-expressed lectin molecules, a macrophage-restricted C-type lectin, MARVIN (Balch et al. 1998) and a functional β-glucan receptor previously reported as a DC-restricted molecule, Dectin-1 (Brown and Gordon 2001; Willment et al. 2001). Currently the best candidate that we have for a pan macrophage marker is CD68, a member of the lysosomal associated membrane protein (LAMP) family (Holness and Simmons 1993). Anti CD68 monoclonal antibodies recognise tissue-resident macrophages in a wide range of normal human tissues and reveal the presence of activated macrophages in human pathologies such as atherosclerosis and arthritis (Pulford et al. 1990). The FA/11 monoclonal antibody recognises the mouse homologue of human CD68, macrosialin (the product of the murine *Cd68* gene). FA/11 detects a similarly broad range of tissue resident macrophages in murine tissues (Rabinowitz and Gordon 1991). For all of the candidate macrophage-restricted markers that we have mentioned in this section, mRNAs and proteins expressed by these genes can be found in some non-myeloid cell types in certain tissues, e.g. SR-A expression in hepatic endothelium or MMR expression in placenta. The term "macrophage-restricted" should be seen as a relative rather than an absolute description for the pattern of expression of these genes. Strikingly, there have been no reports of any macrophage-restricted or even myeloid-restricted transcription factors to date. Many macrophage-expressed gene promoters contain binding sites for the ETS family transcription factor PU.1, but this helix-loop-helix transcription factor is also expressed in B lymphocytes and granulocytes and has been implicated in eosinophil gene expression (Chen et al. 1995; van Dijk et al. 1998).

1.2 Potential Applications of Macrophage-Specific Gene Targeting

If we could develop gene expression systems that would allow us to reproducibly direct high-level transgene expression to macrophages in vivo, this genetic technology would find multiple applications both in basic science and, potentially, in clinical medicine.

Successful macrophage gene targeting would allow us to test the effect of specific transgenes on macrophage function in normal animals. These studies could be extended to study the role of macrophages in animal models of important human pathologies such as atherosclerosis, arthritis and neuroinflammation as well as infectious disease models. One potential application for lineage-specific gene targeting would be the development of lineage-specific gene knockout animals. Macrophage-restricted expression of the Cre recombinase would allow the excision of a "phloxed" allele from the genome only in macrophages. The first reports of such a myeloid-restricted excision strategy used mice in which the Cre recombinase was recombined into the murine lysozyme gene. When crossed with two different strains of mice carrying loxP-flanked genes, highly efficient

excision was seen in both granulocytes and mature macrophages (Clausen et al. 1999). Clearly using the lysozyme locus does not direct gene excision targeted only to macrophages.

A recently developed technology for interfering with gene expression is post-transcriptional gene silencing induced by RNA interference or RNAi. This technique has been successfully used to study gene function in invertebrates and plants and recent technical developments may make this system more useful for interfering with mammalian gene expression (Caplen et al. 2002; Paddison et al. 2002). The RNAi technique relies on expressing specific double-stranded RNAs in specific cell types. Obviously, provision of macrophage-specific expression could allow for macrophage-specific ablation of selected genes.

One potential clinical application for high-level macrophage-specific gene expression would be somatic gene therapy for inherited metabolic diseases. One obvious candidate for macrophage gene therapy would be amelioration of the debilitating effects of inherited lysosomal storage disorders including Gaucher disease, Fabry disease and Niemann-Pick disease. This very heterogeneous group of metabolic diseases has been an attractive target for the development of somatic gene therapy protocols because of the paucity of good therapeutic options for afflicted individuals (currently limited to bone marrow transplantation and costly enzyme-replacement therapy). In Gaucher disease, the enzyme glucocerebrosidase is absent from all cells leading to an intracellular accumulation of undigested glucosylceramide, particularly affecting macrophages. Retroviral gene transfer of the glucocerebrosidase gene can correct the enzyme deficiency in Gaucher disease cell lines (Aran et al. 1996; Schuening et al. 1997) and significant levels of glucocerebrosidase have been obtained in transgenic animals (Guy et al. 1999). Retroviral gene delivery via $CD34^+$ bone marrow cells transduced ex vivo has been attempted in a pilot study of three patients. After reintroduction into patients, retrovirus-infected cells could be detected for up to 3 months, but the very low efficiency of gene correction (~0.2% of cells) and the low levels of glucocerebrosidase expression were insufficient to make any impact on the course of the disease (Dunbar et al. 1998). The macrophage could also be a useful vehicle for production of secreted proteins absent in the plasma of inherited diseases such as factor VIII in haemophilia A or factor IX in haemophilia B. Alternatively, targeting therapeutic gene expression to the alveolar macrophage could be an attractive therapeutic modality for respiratory diseases including emphysema and cystic fibrosis.

Given the central role of macrophages in innate immunity and their ability to present antigen in the context of MHC class II, efficient macrophage and DC gene targeting would allow the development of new protocols for immunomodulation. Such protocols could involve either in vivo delivery via a "gene gun" apparatus or ex vivo transfection of macrophages or DCs with a view to eliciting a strong adaptive immune response against tumour-specific antigens. Several recent reports have shown successful vaccination against HIV and malaria using a combination of DNA vaccine priming followed by recombinant virus booster. Nearly all DNA vaccination has been performed using bacterial plasmids that

use the human cytomegalovirus (CMV) immediate early promoter/enhancer. To date there have been very few reports of using promoters that would direct high-level gene expression specifically in macrophages or DCs. While broad cell-type specificity might be suitable or preferable for local antigen delivery, the ability to direct gene expression specifically to Langerhans cells in vivo might allow for the manipulation of antigen-presenting cell biology through expression of specific cytokines, co-receptors or chemokine receptors. Other potential approaches to immunotherapy might be to use macrophage-specific gene expression to deliver therapeutic doses of anti-inflammatory cytokines such as interleukin (IL)-10 (Lang et al. 2002).

1.3
A Very Brief Introduction to Mammalian Gene Regulation

Before discussing how macrophage-specific gene expression programmes are established, it is worth briefly reviewing current ideas about mammalian gene regulation. Differential gene expression in mammalian cells appears to be regulated primarily at the level of transcription initiation. For this reason, studies of gene expression have focussed on DNA–protein interactions that lead to the productive engagement of RNA polymerase II with the transcription initiation sites of mammalian genes. The DNA sequences immediately 5′ of the major transcription initiation site constitute the gene's promoter and contain a number of recognisable sequence motifs including CCAAT boxes, GC sequences and TATA boxes. Nearly all the macrophage-restricted genes studied to date do not conform to the typical mammalian promoter organisation in that they do not contain recognisable TATA-box sequences within their promoters. Instead they contain one or several purine-rich DNA sequences containing the motif GGAA around the transcription initiation site (Tenen et al. 1997; Clarke and Gordon 1998). For many macrophage-expressed genes, these GGAA sites have been shown to bind the ETS family transcription factor PU.1, and mutations that abolish PU.1 binding markedly reduce promoter activity. PU.1 is not a macrophage-specific transcription factor, however, as PU.1 binding sites have been shown to be important for expression of genes in B lymphocytes and eosinophils, and PU.1 is also expressed in neutrophils (Chen et al. 1995). PU.1 has been shown to interact with a number of transcription factors including AML-1, CCAAT enhancer-binding proteins (C/EBPs), interferon regulatory factor (IRF)-4 and microphthalmia transcription factor (MITF). These combinatorial interactions with other classes of transcription factor may act to restrict PU.1's transcription enhancing activity to cells of the macrophage lineage. Transcription factors bound to the promoter and more distant enhancer elements interact with a cohort of other nuclear proteins to form a stable pre-initiation complex containing RNA polymerase II. These large multi-protein complexes contain classes of proteins called coactivators and corepressors that play a key role in mammalian gene regulation (Rosenfeld and Glass 2001).

For several genes that are expressed in other haematopoietic cell types, compelling evidence has been presented that sequences residing up to 50 kbp away from the gene can exert a powerful influence on gene expression. These sequence elements, termed locus control regions (LCRs), are especially important in allowing genes to escape the repressive effects of assembly into inactive chromatin. The first LCR to be identified was associated with a series of DNase I hypersensitive sites found in the chromatin of the human β-globin locus (Grosveld et al. 1987). When these LCR sequences were incorporated into DNA used for microinjection of mouse embryos, the resultant transgenic mice were shown to express human β-globin transgenes at a high level only in cells of the erythroid lineage. Importantly, the human β-globin transgenes were expressed at the same level as the endogenous murine β-globin genes (Blom van Assendelft et al. 1989; Talbot et al. 1989). The functional definition of an LCR sequence is that it can direct the position independent expression of a transgene and that the observed levels of transgene expression are directly proportional to copy number (Orkin 1990). LCRs were subsequently described in the human T-cell gene CD2, the human T-cell receptor gene locus and at least two gene loci that direct expression in B cells (Greaves et al. 1989; Diaz et al. 1994; Madisen and Groudine 1994; Sabbattini et al. 1999; reviewed in Grosveld 1999; Dillon and Sabbattini 2000; Festenstein and Kioussis 2000).

2 Macrophage-Specific Gene Targeting in Transgenic Animals

In Table 1 we have listed published reports of successful targeting of heterologous transgenes to macrophages in vivo. In the following section of our review we will briefly discuss published reports of macrophage gene targeting in transgenic mouse experiments.

Table 1 Examples of successful targetingof transgene expression to macrophages in transgenic animals

Transgene	Targeting sequence	Reference
Scavenger Receptor SR-AIII	CD68 promoter and IVS1	Gough et al. 2001
Interleukin-10	CD68 promoter and IVS1	Lang et al. 2002
Murine Abq MHC class II	CD68 promoter and IVS1	Unpublished data
Matrix metalloproteinase-1	Human SR-A promoter	Lemaitre et al. 2001
Hormone-sensitive lipase	Human SR-A promoter	Escary et al. 1999
Lipoprotein lipase	Human SR-A promoter	Wilson et al. 2001
Human apoA1	Human SR-A promoter	Major et al. ApoAI
Human growth hormone	Human SR-A promoter	Horvai et al. 1995
Bovine scavenger receptor SR-A	Chicken lysozyme minigene	Daugherty et al. 2001
Interferon-γ receptor dominant negative mutant	Human lysozyme gene	Dighe et al. 1995
Glucocerebrosidase	Murine MHC Class II LCR	Guy et al. 1999
PML/RARalpha	CD11b promoter	Early et al. 1996

2.1 The Human CD11b Promoter

CD11b is an integrin subunit expressed by monocytes, most macrophage populations, granulocytes, natural killer cells and a subset of $CD5^+$ B lymphocytes. Daniel Tenen and colleagues published a series of reports in which they characterised the promoter of the human CD11b gene and identified sequences that directed reporter gene expression in transiently transfected myeloid cell lines. Key elements within the human CD11b promoter included a PU.1 binding site at position 20 and an Sp1 binding site at position 60 (Pahl et al. 1992; Chen et al. 1993; Pahl et al. 1993).

A 1.8 kbp fragment of the human CD11b promoter was tested for its ability to direct macrophage-restricted expression of two different reporter genes, the *Escherichia coli* β-galactosidase gene and a murine Thy1.1 CDNA with human growth hormone introns and polA addition sequence (Dziennis et al. 1995). One line of transgenic mice was established carrying the Thy 1.1 transgene, and two lines of transgenic mice were established carrying the β-galactosidase transgene. Northern blot and RNase protection analysis demonstrated transgene expression in elicited peritoneal macrophages and fluorescence-activated cell sorter (FACS) analysis with a Thy 1.1-specific monoclonal antibody showed that ~50% of peritoneal macrophages expressed the Thy 1.1 transgene. Two-colour FACS analysis using the B-cell specific marker B220 and the granulocyte-specific reagent Gr1 showed that the Thy1.1 transgene was expressed in granulocytes and B cells as well as a subpopulation of $Mac1^+$ macrophages (Dziennis et al. 1995).

In a separate study, Back et al. used a 1.5-kbp fragment of the CD11b promoter to direct expression of a human CD4 reporter gene in transgenic mice (Back et al. 1995). In three independent lines of transgenic mice Back et al. saw no expression in macrophages and detected human CD4 transgene expression in granulocytes and B lymphocytes in two of the three lines of mice (Back et al. 1995). The discrepancy in the expression pattern reported by the two groups could be caused by the relatively small number of transgenic lines studied or differences in the design of the transgenic targeting construct used, notably the provision of human growth hormone gene sequences in the CD11b Thy 1.1 construct.

In the only other published study in which the human CD11b promoter was used to direct transgene expression, Early et al. reported the phenotype of transgenic mice expressing a PML/RARα transgene using the CD11b expression cassette developed by the Tenen laboratory. In one line of founder mice, transgene expression was detected in bone marrow by RT-PCR, but no experiments were performed to demonstrate which haematopoietic cell type was expressing the transgene (Early et al. 1996). In summary, published reports show that the CD11b promoter can direct transgene expression to myeloid cell types, but the efficiency and specificity of targeting to macrophages in vivo leave much to be desired.

2.2
Lysozyme-Directed Transgene Expression

The enzyme lysozyme degrades components of the bacterial cell wall. In mammalian species, lysozyme is expressed by cells of the innate immune system especially neutrophils and activated macrophages and by the Paneth cells of the intestine. In the mouse genome there are two lysozyme genes, the M lysozyme gene that directs lysozyme synthesis in neutrophils and macrophages while the linked P lysozyme gene is expressed specifically in the Paneth cells of the intestine (Cross et al. 1988). In contrast, there is only one lysozyme gene in the human genome. The transcriptional regulatory sequences of the murine M lysozyme gene have been studied in transient transfection assays. Sequences capable of directing expression in myeloid cells have been identified in the promoter and an enhancer element identified in the 3′ flanking sequences of the M lysozyme gene (Mollers et al. 1992). The only report of using murine lysozyme sequences to target myeloid cell expression in vivo is the report of Clausen et al. who integrated a copy of the Cre recombinase into the murine M lysozyme gene by homologous recombination and demonstrated myeloid restricted excision of P lox flanked target genes in two different strains of transgenic mice (Clausen et al. 1999). Lysozyme Cre mice have recently been used to generate transgenic mice that have macrophage-specific ablation of the IL-4 receptor α chain (F. Brombacher, personal communication).

Work from our own laboratory showed that a 3.5-kbp fragment of the human lysozyme gene was able to direct expression of a bacterial reporter gene, chloramphenicol acetyltransferase (CAT), to myeloid cells in three independent lines of transgenic mice. Transgene activity was highest in bone marrow, spleen, lung and thymus, and CAT enzyme activity was detectable in elicited granulocytes and macrophages (Clarke et al. 1996). The same 3.5-kbp human lysozyme promoter fragment was used to direct myeloid-restricted expression of a dominant negative mutant of the interferon-γ receptor alpha chain (Dighe et al. 1995). We have demonstrated that human lysozyme promoter sequences can target gene expression to immature and mature myeloid cells, but our experience is that the levels of transgene mRNA expression are low and expression is confined to a subset of tissue macrophages in vivo.

The chicken lysozyme gene has been used as a model system for studying changes in chromatin organisation related to gene expression. Additionally, the ability of different fragments of the chicken lysozyme gene locus to direct position-independent transgene expression has been tested in stably transfected cell lines and transgenic animals (reviewed in Bonifer et al. 1997). The initial experiments of Stief and Sippel showed that inclusion of chicken lysozyme gene sequences with the properties of matrix attachment regions (MARs) in reporter gene plasmids gave position-independent expression in stably transfected mammalian cell lines (Stief et al. 1989). This property of chicken lysozyme gene sequences was confirmed in the experiments of Bonifer et al., who used a 21-kbp DNA fragment encompassing the chicken lysozyme gene locus and its associat-

ed DNase I hypersensitive sites and MARs to generate transgenic mice. The chicken lysozyme gene was expressed in mature myeloid cells, including macrophages, in a copy-number dependent manner regardless of the site of integration into the mouse genome (Bonifer et al. 1990, 1997; Bonifer et al. 1994). Chicken lysozyme MAR sequences have been used as a component of several eukaryotic gene expression vectors used for stable transfection experiments in mammalian cells (Zahn et al. 2001) and even in zebrafish transgenesis (Caldovic et al. 1999).

We are aware of only one report in which chicken lysozyme gene sequences have been used to express a heterologous gene in the macrophages of transgenic mice. Daugherty et al. recently reported the generation of transgenic mice in which a bovine scavenger receptor bSR-A was expressed under the control of the chicken lysozyme promoter. The authors detected transgene mRNA in elicited macrophages but they were unable to detect expression of bSR-A protein due to a lack of specific antibodies. The bSR-A mice were shown to have altered peritoneal macrophage adhesion in vitro and enhanced granuloma formation in vivo, both properties that would be consistent with enhanced scavenger receptor expression. In the absence of transgene protein expression data, it is hard to evaluate the usefulness of the chicken lysozyme cassette for directing macrophage-specific expression in transgenic mice.

2.3 Regulatory Elements of the Human *c-fes* Gene

The *c-fes* proto-oncogene encodes a 92-kDa protein tyrosine kinase that is associated with the common β-chain subunit of the granulocyte-macrophage (GM)-CSF and IL-3 receptors. A 13.2-kbp fragment of human genomic DNA containing all 19 exons and 18 introns of the *c-fes* gene together with 446 bp of 5′ flanking and 1.5 kb of 3′ flanking sequence was used to generate transgenic mice (Greer et al. 1990). The human *c-fes* transgene mRNA showed the same pattern of expression as the endogenous mouse *c-fes* gene, being expressed at high level in bone marrow-derived macrophages. The authors reported that the 13.2-kbp fragment of DNA containing the human *c-fes* gene was able to confer position-independent expression that was proportional to copy number and that the human *c-fes* transgene appeared to be expressed as efficiently as the endogenous *c-fes* gene (Greer et al. 1990).

Heydemann et al. made a series of plasmid constructs in which different fragments of the 13.2-kb *c-fes* gene fragment were tested for their ability to direct myeloid-specific gene expression in transgenic mice. Sequences important for transgenic expression were shown to reside in introns 1 and 3 of the human *c-fes* gene (Heydemann et al. 2000). On the basis of these observations, the authors concluded that sequences within introns 1 and 3 constitute a myeloid-specific locus control region (LCR). In the same paper, the authors went on to explore the ability of the human *c-fes* LCR to direct myeloid expression of a heterologous enhanced green fluorescent protein (EGFP) transgene. EGFP transgene

expression was only detected in 3 of the 8 transgenic lines analysed. Transgene expression was analysed by flow cytometry and EGFP fluorescence was detected in 50% of $Gr1^+$ granulocytes and 50% of $Mac1^+$ myeloid cells but absent in $B220^+$ B lymphocytes and absent in thymocytes (Heydemann et al. 2000). The failure of the *c-fes* LCR to give a higher efficiency of heterologous transgene expression in myeloid cells is surprising. Given that *c-fes* LCR sequences are completely contained within the *c-fes* gene it is possible that these sequences need to be included in the primary transcription unit for maximal effect.

2.4
Use of the Human SR-A Gene Promoter to Target Gene Expression in Transgenic Mice

Macrophage scavenger receptors mediate the uptake of modified forms of LDL. The first macrophage scavenger receptor gene to be cloned was SR-A, which gives rise to at least two different functional isoforms of the receptor, SR-AI and SR-AII, by alternative splicing (Emi et al. 1993). SR-A is expressed by a subset of tissue-resident macrophages in vivo, such as alveolar macrophages and Kupffer cells, as well as macrophages in inflammatory pathologies, such as atherosclerosis (Gough et al. 1999). The promoter of the human SR-A gene has been analysed by a number of laboratories in transient transfection assays. The laboratory of Christopher Glass identified SR-A promoter sequences between positions 245 and +46 as being important for expression in the myeloid leukaemia cell line THP-1 and implicated the transcription factors AP1, PU.1 and ets-2 as being important for SR-A promoter activity (Wu et al. 1994). Further analysis of the SR-A promoter by the Glass laboratory identified an enhancer sequence between positions 4500 and 4100 (Moulton et al. 1994). A human SR-A promoter/enhancer cassette containing human SR-A promoter sequences between 245 and +46 fused to the upstream enhancer sequence was used in transgenic experiments. Horvai et al. first demonstrated the ability of this SR-A enhancer/promoter cassette to direct macrophage-restricted expression of a human growth hormone reporter gene in macrophages of transgenic mice and demonstrated transgene expression by bone marrow-derived macrophages cells differentiated in the presence of M-CSF (Horvai et al. 1995) The current literature furnishes several examples of successful targeting to macrophages using the SR-A enhancer/promoter cassette developed by the Glass laboratory (Table 1).

2.5
Regulatory Sequences of the Human CD68 Gene

CD68 is probably the best pan macrophage marker in immunohistochemistry studies of human tissues (Pulford et al. 1990). The protein recognised by CD68 monoclonal antibodies is a member of the lysosome-associated membrane protein (LAMP) family (Holness and Simmons 1993) and human CD68 shows 72% amino acid identity with the murine macrosialin protein (Holness et al. 1993).

Li et al. have studied the elements of the murine macrosialin promoter required for macrophage-specific expression (Li et al. 1998) and our laboratory has characterised the sequences responsible for transcriptional regulation of the human CD68 gene (Greaves et al. 1998). The draft human genome sequence shows that the human CD68 gene is one of 9 genes in a ~140 kbp region of chromosome 17p13 and the CD68 gene ATG initiation codon lies 669 bp 3′ of the EIF4A1gene, which encodes eukaryotic initiation factor 4AI (Jones et al. 1998).

The 666-bp EIF4A1 CD68 intergenic region has significant promoter activity in murine macrophage cell lines and a number of other cell types (Greaves et al. 1998). A 5′ deletion analysis of the CD68 5′ flanking sequence showed that a promoter fragment of only 150 bp directs reporter gene expression in transiently transfected macrophage cell lines to a level that is twice that seen using the SV40 enhancer in the same reporter plasmid. We cloned each of the five CD68 gene introns 3′ of a minimal CD68 promoter and showed that only intron 1 was able to contribute to macrophage-specific expression (Greaves et al. 1998). Taken together, these observations led us to conclude that the combination of the human CD68 promoter and the 83-bp first intron of the human CD68 gene cooperate to direct high-level reporter gene expression in transiently transfected macrophage cell lines.

To test the utility of human CD68 gene sequences in macrophage gene-targeting experiments, we have generated a series of CD68 expression vectors in which cDNA fragments of different transgenes have been cloned downstream of a 2940 CD68 promoter fragment and the 83-bp first intron of the CD68 gene. Initial experiments using splice variants of the human macrophage scavenger receptor SR-A showed that human CD68 gene sequences were able to direct high-level expression of SR-A transgenes in stably transfected RAW cells (Gough et al. 2001). In two lines of transgenic mice, we were able to demonstrate high levels of a SR-AIII transgene in bone marrow and elicited peritoneal macrophages (Gough et al. 2001). More recently we have shown the utility of the CD68 promoter intron 1 cassette to direct macrophage-specific expression of an IL-10 transgene in transgenic mice. Macrophages of these transgenic mice constitutively express high levels of the deactivating cytokine IL-10 and display profound changes in their response to bacterial pathogens. No transgene expression is detected in neutrophils, B cells or T cells (Lang et al. 2002). These initial experiments suggest that CD68 transcriptional regulatory sequences may be useful for directing transgene expression in cells of the mononuclear phagocyte lineage. One reason for the success of the CD68 vector may be the inclusion of the CD68 intron close to the 5′ end of the transgene.

3
Gene Delivery to Macrophages Using Viral and Non-viral Vectors

3.1
Retroviral Vectors

Retroviruses have been used widely in gene therapy protocols and as a vehicle for gene delivery to primary cells, including bone marrow progenitor cells. First generation retroviral vectors were of limited usefulness in macrophage transduction because they were only able to deliver transgene expression in dividing cells. More recently, developed recombinant lentiviral vectors have shown great promise in gene delivery to a wide range of cell types in vivo (Somia and Verma 2000). The most spectacular example of the power of this technology is shown by expression of GFP in every tissue of an adult mouse following transduction of single-cell mouse embryos with a recombinant lentiviral vector (Lois et al. 2002). The majority of retroviral vectors rely on viral or housekeeping gene promoters to direct transgene expression. However, a recent paper by Cui et al. compared the efficiency of self-inactivating lentiviral vectors containing the promoter of the housekeeping gene EF-1α or the class II gene HLA-DRα to drive GFP expression in antigen-presenting cells following infection of human $CD34^+$ bone marrow-derived progenitor cells. The HLA-DRα promoter efficiently targeted GFP expression to differentiated DCs in non-obese diabetic, severe combined immunodeficiency (NOD/SCID) mice engrafted with human haematopoietic stem cells (Cui et al. 2002). This report shows the feasibility of developing myeloid-restricted transgene expression in vivo through development of retroviral vectors containing macrophage or DC-specific promoters.

3.2
Adenovirus-Mediated Gene Transfer

Recombinant adenoviruses offer several significant advantages over retroviral vectors for gene delivery, notably the ability to routinely prepare high titres of recombinant virus and the ability to efficiently infect non-dividing cells. Recombinant adenoviruses have been used to drive heterologous gene expression in human macrophages and DCs cultured ex vivo. Examples include the restoration of respiratory burst in monocyte-derived macrophages from patients with X-linked chronic granulomatous disease using a recombinant adenovirus encoding the gp91 phox subunit (Schneider et al. 1997), MUC1 gene transduction of human blood-derived DCs (Maruyama et al. 2001) and modification of murine DCs to secrete the CC chemokine macrophage-derived chemokine (MDC) (Kikuchi and Crystal 2001). There are also reports of recombinant adenovirus transduction of tissue resident macrophages in vivo, for instance the study of Wheeler et al., which demonstrated transgene expression in Kupffer cells in mice infected with a recombinant adenovirus (Wheeler et al. 2001). Not all tissue-resident macrophages are equally susceptible to adenovirus infection due to

differences in the level of expression of the cell-surface integrins that mediate adenoviral gene entry into cells. It has been reported that alveolar macrophages are refractory to adenovirus infection and that this block to infection is not alleviated by treatment with M-CSF (Kaner et al. 1999; Conron et al. 2001). One approach that might be adopted to circumvent this obstacle to in vivo gene delivery would be the development of recombinant adenoviral vectors with altered viral coat proteins (Wickham 2000).

For experiments where primary cells are manipulated ex vivo there is no strict requirement for including a macrophage or DC-specific promoter in the adenovirus vector. However, the utility of recombinant adenoviruses or adeno-associated adenoviruses for in vivo gene delivery could be greatly enhanced by developing adenoviral vectors containing macrophage-specific promoters. We have made a recombinant adenovirus that uses the human CD68 promoter to drive expression of a soluble form of the human scavenger receptor (Wickham 2000) and we are developing recombinant viral vectors that include both the CD68 promoter and the macrophage-specific enhancer element within the first intron of the CD68 gene.

3.3 Non-Viral Vectors

A number of different compounds have been developed that facilitate DNA entry into cultured cells in vitro, and some of these compounds have been shown to mediate DNA delivery in vivo. Peritoneal macrophages have been shown to take up naked plasmid DNA via a non-scavenger receptor-mediated mechanism, although this process is very inefficient (Takakura et al. 1999). One approach to directing DNA delivery specifically to macrophages uses potential ligands for known macrophage receptors. The first example of receptor-mediated gene transfer to macrophages used DNA coupled to a ligand for the macrophage mannose receptor (Ferkol et al. 1996). Attempts to use this technology to deliver an alpha1 antitrypsin gene to alveolar macrophages in vivo were very inefficient (Ferkol et al. 1998). Recent papers have used mannose coupled to polyethylenimine (PEI) to deliver DNA to DCs (Diebold et al. 1999; Diebold et al. 2001) and Kawakami et al. have explored the possibility of using mannosylated, fucosylated and galactosylated liposome-DNA complexes for macrophage-specific gene delivery (Kawakami et al. 2000a,b).

One interesting approach to in vivo protein, drug or DNA delivery to selected cell populations has been developed by the laboratory of Seppo Ylä-Herttuala. Lehtolainen et al. constructed a novel endocytic receptor in which the C-terminal ligand-binding domain of the bovine scavenger receptor has been replaced with avidin. This "scavidin" receptor behaves as a novel endocytic receptor that binds and internalises biotinylated molecules (Lehtolainen et al. 2001). Macrophage-restricted expression of this or similar novel receptors could allow for highly selective delivery of therapeutic compounds to macrophages in vivo.

4 Future Prospects

Significant progress has been made towards identifying important regulatory elements for macrophage-specific gene expression but we are still some way from having a macrophage expression vector that will reproducibly yield high-level expression in macrophage populations in vivo. The identification of potential locus control regions in the human *c-fes* and the murine *spi-1* genes is an exciting development in the field and analysis of these regions may reveal important molecular mechanisms that underlie the development of the mononuclear phagocyte lineage. It will be very interesting to see if these sequences can be used to develop macrophage-specific expression vectors.

Table 1 shows that the human SR-A promoter has found the most widespread application in directing macrophage-specific expression in transgenic mice so far, and the usefulness of the SR-A promoter may be augmented by changes in vector design such as the introduction of heterologous introns into the primary transcription unit and careful selection of poly A addition sequences (C. Glass, personal communication). Most of the experiments we have discussed have used pronuclear microinjection of naked DNA to transfer transgene expression constructs to the mouse germline. So far there have been few reports of attempts to incorporate macrophage-specific promoters into viral vectors that would allow for macrophage-specific expression following transduction of bone marrow-derived stem cells or haematopoietic progenitor cells.

All the examples of macrophage gene targeting that we have discussed in this review have been aimed at directing constitutive transgene expression in macrophages. Recently we have used human CD68 sequences to drive IL-10 expression in transgenic mice, and mice with more than two copies of the transgene had very high serum levels of IL-10 and died from opportunistic bacterial infections (Lang et al. 2002). This observation suggests that for some transgenes it will be important to start designing vectors that allow for inducible macrophage gene expression. Currently, inducible gene expression systems based on the bacterial Tet repressor and the tetracycline analogue doxycycline seem to offer the best prospects for mediating regulated gene expression in transgenic mice (Lewandoski et al. 2001). The development of an inducible macrophage expression system would allow a detailed appreciation of macrophage function in a whole range of pathological settings, ranging from endotoxic shock to atherosclerosis.

Impressive preliminary results have been obtained using naked DNA to induce humoral and cellular immune responses to test antigens in animal systems. The success obtained using DNA vaccine prime, recombinant virus boost vaccination protocols has focused attention on generating specific immune responses to candidate antigens derived from infectious disease agents or tumours (Hanke et al. 1999). The ability to target macrophage and DC populations in vivo could have very important consequences for our ability to develop immunomodulation protocols for therapeutic benefit. Of especial interest would be switching a

predominantly Th2-type immune response to a more Th1-type immune response in allergic asthma or the induction of immune tolerance in transplantation or autoimmunity.

It is our belief that progress in understanding the basic biology of macrophages will yield benefits in developing new treatments for pathologies characterised by monocyte recruitment, macrophage differentiation or macrophage dysfunction such as atherosclerosis, arthritis and lysosomal storage disorders.

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Part 2
Macrophage Targets in Inflammation

Integrins of Macrophages and Macrophage-Like Cells

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Abstract The integrins are a family of heterodimeric adhesion receptors present on virtually every cell in metazoan organisms. Macrophage integrins are involved in adhesion to extracellular matrix and to other cells, in phagocytosis, and in cell migration and spreading. Macrophage integrins also transduce signals from the extracellular environment, both through activation of specific kinase cascades and through modulation of cytoskeletal elements. The ligand-binding ability of macrophage integrins can be regulated by environmental cues including growth factors, lipid mediators, bacterial peptides, and fragments of complement, clotting, and other proteins that may accumulate at sites of inflammation. In addition, integrins can function as components of multimolecular plasma membrane complexes that include tetraspanins, proteases, and other receptors. These multimolecular complexes can be endowed with functional properties not inherent in the isolated components. This review summarizes current understanding of the complex biology of macrophage integrins. The involvement of integrins in many macrophage processes fundamental to the function of these cells for inflammation and host defense makes them double-edged swords as candidates for therapeutic intervention. Beneficial effects of broad in-

tegrin blockade in ameliorating idiopathic inflammation may be accompanied by unacceptable susceptibility to infection or other deleterious side effects. However, more specific blockade of less widely expressed integrins holds the promise of a better therapeutic effect. For example, recent studies in animals suggest potential therapeutic use for blockade of osteoclast $\alpha V\beta 3$ in osteoporosis and of T-cell and monocyte $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in arthritis. Further understanding of the molecular mechanisms through which macrophage integrins control key events in a variety of diseases may lead to the development of inhibitors of even greater specificity. The integrins remain appealing therapeutic targets because of their central role in macrophage biology.

Keywords Adhesion, Affinity modulation, Cytoskeleton, Dietary fatty acids, Extracellular matrix, ICAM-1, Macrophages, Migration, Phagocytosis, Signal transduction, VCAM-1

The integrins are a family of 22 adhesion receptors that recognize a wide variety of ligands, including: various proteins and proteoglycans of basement membranes and extracellular matrix; members of the immunoglobulin superfamily on cell surfaces; and potentially some anionic phospholipids. Integrins are present on all nucleated cells and on platelets; only erythrocytes are apparently devoid of integrins, although relatively mature erythrocyte precursors do express these receptors. Integrins are involved in leukocyte proliferation, maturation, migration, activation, and multiple effector functions including phagocytosis, cytotoxicity, synthesis of cytokines, and activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Based on the importance of integrins in leukocyte migration to and activation at sites of inflammation, these receptors have for some time been considered excellent targets for therapy of inflammatory diseases. Recently, understanding of the critical role of osteoclast integrins has led to great enthusiasm for targeting these integrins to prevent or treat osteoporosis. After a brief review of general aspects of integrin structure and function, this review will consider specifically macrophage integrins and the physiologic and pathologic processes in which they are involved, and ultimately the potential therapeutic applications of anti-integrin therapy to treat diseases in which macrophages and related cells may have an important role.

1 Integrin Structure

All integrins are heterodimers of two type I membrane proteins (N terminal on the extracytoplasmic face of the membrane) that function primarily as adhesion receptors. There are 17 known α chain proteins ($\alpha 1$–$\alpha 11$, αM, αL, αX, αD, αV, αE) and 8 known β chain proteins ($\beta 1$–$\beta 8$); various α and β chains combine in the endoplasmic reticulum to create a family of 22 cell surface receptors. The interactions between the two chains are complex, and failure to associate appropriately leads to failure of secretion. Recently, the crystal structure of the inte-

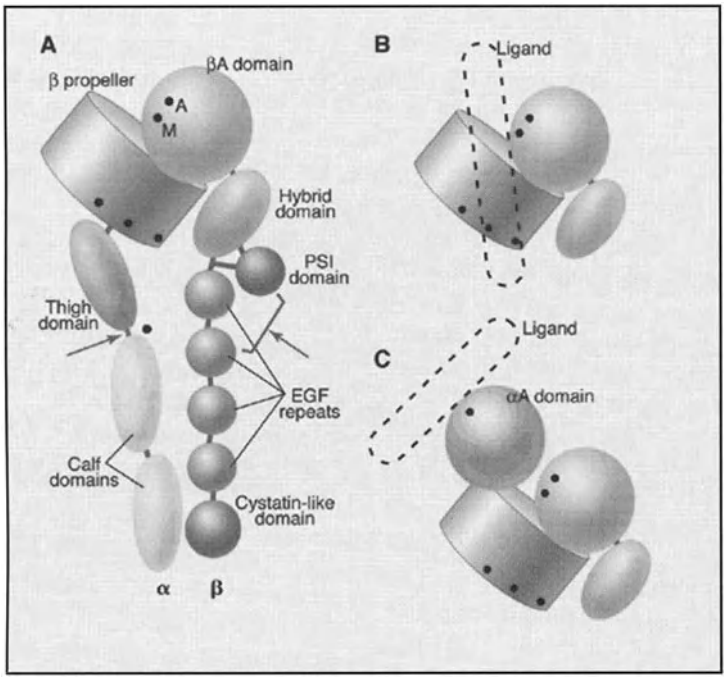

Fig. 1A–C Integrin structure. **A** Structure of integrin $\alpha V\beta 3$ based on the crystallographic data from Xiong et al. (2001). **B** The extensive contact between the β propeller domain of the α chain and the βA domain of the β chain provides the interface for ligand binding. **C** For integrins that contain an I domain (αA domain, **C**) in the α chain, this domain likely interacts with the α/β interface created by the β propeller and the βA domain and provides an independent ligand binding face. (Reproduced from Humphries and Mould 2001, with permission)

grin $\alpha V\beta 3$ was solved (Xiong et al. 2001). A key feature of the structure is a previously predicted "beta-propeller" domain in the α chain with heptad symmetry, reminiscent of the structure of the beta chain of heterotrimeric G proteins (Springer 1997). The aminoterminal domain of the β chain (the so-called βA domain) interacts with this beta propeller structure and has a fold similar to the alpha chain of heterotrimeric G proteins, as well as to domains in a variety of extracellular molecules, including von Willebrand factor. The interface between the beta propeller and the βA domain closely resembles the interface between alpha and beta chains in heterotrimeric G proteins. Ligands for $\alpha V\beta 3$ are thought to bind at this interface between α and β chains (Fig. 1); this has been confirmed very recently for the peptide ligand Arg-Gly-Asp (Xiong et al. 2002). Carboxyterminal to the βA domain are four highly disulfide bonded epithelial growth factor (EGF)-like domains which also participate in interaction with the α chain (Fig. 1). Some α chains have an additional domain with high homology to the βA domain, also called the A or I (for inserted) domain, which is inserted between spokes 2 and 3 of the beta-propeller structure. When this α chain I do-

main is present, it accounts for most if not all ligand binding by the integrin (Fig. 1). Integrin α chains containing this domain include α1, α2, αL, αM, αX, and αD. Historically and experimentally, an important feature of this domain is its ability to fold on its own, allowing important insights into integrin function.

When the first I domain (from αM) was crystallized, two different structures were noted which depended on the divalent cation used in the crystallization process. The difference between the two structures was a small change in the divalent cation binding site and a much greater shift in the position of an alpha helix at the carboxyterminus of the domain. It is now clear that these two structures represent conformations which differ in affinity for ligand. This conformation-dependent affinity change can account for at least part of the well-documented ability of integrins to alter their ligand binding properties in response to cell activation ("inside-out signaling"). A caveat to this hypothesis is that it would apply only to those few integrins with α chain I domains. However, if the βA domain, which is structurally homologous to this α chain I domain, can undergo a similar conformational change in response to cell activation, the ability of the βA domain to assume two distinct conformations could go a long way toward explaining cellular regulation of ligand binding for non-I domain-containing integrins. The recent crystal structure of αVβ3 with its peptide ligand shows that ligand indeed can induce conformational changes in the βA domain (Xiong et al. 2002), consistent with this hypothesis about integrin activation. In this respect, the homology of integrins with heterotrimeric G proteins is quite intriguing; it appears that nature has conserved a specific molecular mechanism for communicating cell activation in two quite disparate pathways.

Understanding of the mechanism for regulation of ligand binding by I domain-containing integrins has led to the generation of a unique reagent for inhibition of lymphocyte function associated antigen (LFA)-1 (αLβ2) function. Shimaoka et al. (2001) created a soluble I domain that was locked into the high-affinity conformation by introduction of a disulfide bond at relevant sites in the domain. This constitutively high-affinity receptor had almost 10,000-fold increased affinity for intercellular adhesion molecule (ICAM)-1 compared to wild-type I domain or intact αLβ2 and acted as an inhibitor of αLβ2 function both in vitro and in vivo. To this point, only monoclonal antibodies have been available to inhibit αLβ2 function; the new inhibitor has the advantage that it is smaller and, at least theoretically, more readily diffusible to sites where leukocyte αLβ2 might be engaged outside the vasculature.

2 Integrin Expression in Macrophages and Macrophage-Like Cells

Monocytes and macrophages constitutively express integrins of the β1, β2, and β5 families (Table 1). Of the 12 known β1 integrins, monocytes and macrophages have been shown to express α1, α2, α3, α4, α5, and α6 associated with β1. These cells also constitutively express αL and αM associated with β2. In humans, numerous macrophage populations also express αXβ2 (CDllc/CD18), and

Table 1 Monocyte and macrophage integrins

β chain family	Associated α	Ligands
β1	α1	Collagen
	α2	Collagen, laminin
	α3	Laminin
	α4	Fibronectin, VCAM-1
	α5	Fibronectin
	α6	Laminin
β2	αM	ICAM-1, fibrinogen, others[b]
	αL	ICAM-1, ICAM-2
	αX	Fibrinogen
	αD	ICAM-3
β3	αV	Many RGD-containing proteins
β5	αV	Similar to αVβ3
β7[a]	α4	MADCAM-1, fibronectin
	αE	E-cadherin

[a] For a discussion of ligands for αMβ2, see Brown (1991), Ross and Vetvicka (1993), Ehlers (2000), and Humphries and Mould (2001)

[b] There is a single report of β7 expression on macrophages

expression of this receptor increases during monocyte differentiation to macrophages, but in mouse this ICAM-3 receptor seems to be restricted to dendritic cells. A fourth β2-associated a chain, αD, also seems to be expressed to a greater extent on tissue macrophages than monocytes. Coordinate expression of αX and αD is not surprising in view of their tight linkage (11.5 kbp apart) on chromosome 22. αV is the integrin α chain with the most promiscuous β chain pairings, since it can interact with β1, β3, β5, β6, and β8. On human monocytes and murine bone marrow macrophages, the major αV-containing integrin is αVβ5. Of importance, during the maturation of monocytic cells to osteoclasts, αVβ3 expression increases markedly, and αVβ5 expression diminishes. Indeed αVβ3 is the major osteoclast integrin, and it appears to be important both for migration of osteoclasts to and on bone and for regulation of osteoclast survival (McHugh et al. 2000). Unligated αVβ3 leads to rapid apoptosis (Stupack et al. 2001). β7 integrins (αEβ7 and α4β7), expressed on gut-homing lymphocytes, are not present on blood monocytes, but can be induced during inteferon (IFN)-γ-mediated differentiation (Tiisala et al. 1995). These integrins are present on macrophages in liver, lung, and lymph node sinuses, suggesting that they may be markers of differentiation from monocyte to tissue macrophage.

3 Integrins in Macrophage Biology

There have been numerous reviews of leukocyte integrin function (e.g., Madri and Graesser 2000; Ley 2001; Woods and Shimizu 2001; Bunting et al. 2002), so

this section will only highlight briefly the major areas of relevance to macrophage biology.

3.1 Integrins in Monocyte/Macrophage Extravasation

Integrins can mediate both cell–cell and cell–extracellular matrix adhesions. Integrins are necessary for monocyte migration from blood to tissue, which is necessary for macrophage accumulation at sites of inflammation. Whether monocyte extravasation is necessary for replenishing tissue macrophages during normal homeostasis is less certain. There is evidence both for self-renewal of tissue macrophages and for repletion of this population from the bone marrow (Naito et al. 1996). Essentially all studies of integrin involvement in monocyte extravasation have been done in the context of inflammation. The first point of integrin engagement is during monocyte-endothelial interaction. Firm integrin-mediated adhesion of monocyte to endothelium is required for successful extravasation; the major integrins involved in this appear to be $\alpha L\beta 2$ and $\alpha 4\beta 1$. These integrins appear to have overlapping functions in mediating firm adhesion. $\alpha 4\beta 1$ recognizes vascular cell adhesion molecule (VCAM)-1, an endothelial ligand induced at sites of inflammation. $\alpha L\beta 2$ is known to interact with both ICAM-1 and ICAM-2 expressed on endothelial cells; it has also recently been shown to recognize the endothelial tight junction protein junctional adhesion molecule (JAM)-1 may be important in initiating leukocyte migration through the interendothelial junctions (Ostermann et al. 2002). Presumably, mechanisms of transendothelial migration are similar in maintenance of the tissue macrophage pool in homeostatic conditions; since $\alpha L\beta 2$ can recognize endothelial ligands that do not require inflammation for expression (ICAM-2 and JAM-1), it would be a leading candidate to mediate monocyte-endothelial interaction in this circumstance. Consistent with this, unperturbed $\beta 2$ integrin-deficient mice have decreased numbers of pulmonary dendritic cells and macrophages (Schneeberger et al. 2000). However, the defect is only partial, suggesting that there are alternative pathways for maintenance of the tissue macrophage pool.

Transendothelial migration also is important in dendritic cell migration from tissues to lymph nodes after antigen exposure. In this case, cells must cross the lymphatic endothelium to gain entry into the lymph nodes where they can interact with T cells to initiate an immune response. There is some evidence that this migration may be mediated by $\alpha 4\beta 1$ (Zou et al. 2001), although other integrins may contribute as well (Weiss et al. 2001). $\alpha 4\beta 1$ expression is increased during the dendritic cell differentiation that occurs concomitant with migration back to lymph nodes (Puig-Kroger et al. 2000). "Reverse transmigration" (movement across endothelium from the abluminal to the lumenal side) may be important in dendritic cell entry into the afferent lymphatic circulation in order to migrate to the lymph nodes. In vitro, monocyte maturation to dendritic cells is required for this migration, and the maturation process requires both phagocy-

tosis of antigen and interaction of monocytes with collagen (Randolph et al. 1998). This may implicate integrins not only in the transmigration process but also in differentiation to dendritic cells. However, no investigation of the roles of specific integrins in either reverse transmigration or dendritic differentiation of monocytes has been reported.

3.2 Macrophage Interaction with Extracellular Matrix

In addition to transendothelial migration, macrophage integrins mediate both cell migration through tissue and stable adhesion at sites of inflammation, wound repair, and other perturbations of homeostasis. Macrophages have not to date been a popular model system for study of the mechanisms of cell migration, presumably because they are neither as motile as neutrophils or lymphocytes nor as easy to transfect as fibroblasts. Progress in understanding integrins and cell migration in these other model systems has been rapid and reviewed extensively in recent years (e.g., Eliceiri 2001; van der Flier and Sonnenberg 2001; Woods and Shimizu 2001) and will not be reviewed in detail here. Among the key enzymes involved in integrin-mediated adhesion to and/or migration on extracellular matrix are focal adhesion kinase (FAK), PI 3-kinase, and src family kinases. In fibroblasts, endothelial cells, and T cells, FAK can act as a scaffolding molecule for assembly of the other kinases together with certain key structural molecules, such as paxillin, upon integrin ligation. However, FAK itself is only minimally expressed in macrophages which instead express a homologue called pyk2 (Duong and Rodan 2000). While there is good evidence that pyk2 phosphorylation can be activated by adhesion, whether it serves an analogous role to FAK in macrophage migration remains debated. Despite this caveat, there is excellent evidence that phosphatidyl inositol (PI) 3-kinase and src family kinases are involved in integrin mediated adhesion and migration (Suen et al. 1999; Ridley 2001). This suggests that, despite differences in detail, the basic mechanisms of regulation of integrin function are similar in macrophages and fibroblasts.

A potentially important role for integrin–extracellular matrix interactions in macrophages is to regulate phagocytic function. Interaction with several different extracellular matrix proteins, including fibronectin, collagen, and laminin stimulates phagocytosis via both Fcγ and complement receptors (Pommier et al. 1983; Bohnsack et al. 1985; Newman and Tucci 1990). This activation of phagocytic function occurs in polymorphonuclear leukocytes as well, but may have a more complex regulation in that cell type. The $\alpha V\beta 3$ integrin has a major role in this activation and requires association with a second plasma membrane protein, CD47, to initiate the signal. Interferon-γ treatment can downregulate extracellular matrix-mediated activation of macrophages (Wright et al. 1986), suggesting that immunologically activated macrophages may lose some integrin signaling mechanisms; the molecular basis for this phenomenon is unknown.

3.3 Macrophage Integrins in Macrophage Cell–Cell Interactions

Macrophages and dendritic cells participate in a variety of cell–cell interactions outside the vasculature as an essential aspect of their biological functions. For example, they must interact with other cells of the immune system (T cells and B cells), with epithelial cells in the lung and gut, with virally infected cells as part of the immune response, with tumor cells as part of the innate anti-tumor response, and with bone marrow stromal cells during development. Furthermore, macrophages are important in removal of apoptotic cells during development and in adult life.

Interaction with bone marrow stroma seems to depend on interactions between $\alpha 4\beta 1$ on monocyte precursors and VCAM-1 expressed on stromal cells (Teixido et al. 1992; Harada et al. 1998; Berrios et al. 2001; Hidalgo et al. 2001). $\beta 2$ integrins may contribute to this interaction as well; they are clearly important in interactions with tumor cells, infected fibroblasts, and possibly some epithelial cells that express ICAM-1 (Shang and Issekutz 1998; Rosseau et al. 2000; Chang et al. 2002). However, in the case of macrophage interactions with B and T cells, most of the emphasis in studies to date has been on lymphocyte integrin interactions with macrophage immunoglobulin (Ig) superfamily ligands, both for formation of the immunologic synapse (which involves interaction of T cell $\alpha L\beta 2$ with ICAM-1 on dendritic cells or other antigen-presenting cells) and for T-cell homing to thymus or B-cell homing to lymph nodes. Nonetheless, both B and T cells can express ICAM-1 and ICAM-3, so it seems likely that some contribution to cell–cell interactions may arise from recognition of these lymphocyte Ig ligands by macrophage integrins.

In contrast to the almost exclusive use of $\alpha 4\beta 1$ and $\beta 2$ integrins in these cell–cell interactions, several groups have described a role for αV integrins in macrophage recognition of apoptotic cells (Leverrier and Ridley 2001; Ren et al. 2001). Because of the large number of receptors involved in recognition of apoptotic cells by macrophages (Fadok and Henson 1998), inhibition of αV integrins, either with antibody or arginine-glycine-aspartic acid (RGD) peptides, only leads to about 50% diminution of phagocytosis of apoptotic cells. Nonetheless, transfection of $\alpha V\beta 5$ can confer ability to recognize and ingest apoptotic cells on 293 epithelial cells (Albert et al. 2000), and ingestion is initiated by a signaling pathway reminiscent of the pathway defined by studies of apoptotic cell phagocytosis in *Caenorhabditis elegans* (Henson et al. 2001). Phagocytosis of apoptotic cells is anti-inflammatory because these targets induce macrophage production of transforming growth factor (TGF)-β. It has been reported that ligation of αV integrins leads to TGF-β synthesis (Freire-de-Lima et al. 2000), which would suggest that these integrins are critical to the macrophage response to apoptotic cells, but this view has been challenged recently (Huynh et al. 2002).

4
Integrins and the Macrophage Cytoskeleton

Integrins are named because they integrate the extracellular matrix with the intracellular matrix, or cytoskeleton (Tamkun et al. 1986). There are direct connections between integrin cytoplasmic domains and the actin cytoskeleton, mediated via talin, vinculin, and α-actinin. While no direct links are known between integrins and other components of the cytoskeleton, there is evidence that indirect interactions between microtubules and integrins may regulate integrin avidity in macrophages (Zhou et al. 2001). Perhaps this is unsurprising, given the extensive crosstalk among various structural elements of the cytoskeleton, but the involvement of microtubules in regulating integrin adhesion points to potential new mechanisms of cellular control and new targets for pharmacologic regulation. Moreover, the connection between microtubules and integrins may be more fundamental than initially apparent, since the microtubule polymerization inhibitor colchicine blocks integrin-mediated but not FcγR-mediated phagocytosis (Munthe-Kaas et al. 1976). Possibly, microtubule-dependent delivery of intracellular vesicle membranes is important for integrin regulation.

5
Integrin Signaling in Migration and Phagocytosis

Macrophage migration and spreading on extracellular matrix proteins are dependent on integrin contacts with cytoskeleton. The basic paradigm developed from work in fibroblasts—that different rho family GTPases regulate distinct aspects of cytoskeletal assembly—seems to operate in macrophages as well. cdc42 and rac are apparently important in filapodial and lamellar extension, while rho is involved in the generation of contractility. In macrophage migration, both rac and rho are required for migration, and rac seems to be activated downstream of PI 3-kinase, at least in response to CSF-1 (Ridley 2001). In contrast, the requirement for cdc42 is not for migration but for appropriate sensing of a chemotactic gradient; when cdc42 is inhibited, macrophages migrate randomly (Ridley et al. 1999). One difference between fibroblasts and macrophages is that actin stress fibers are less prominent in macrophages than fibroblasts, and "focal contacts"—the linear arrangement of integrins at adhesion sites in response to the contractile force of the stress fibers—are very much more frequent in fibroblasts. Instead, rhoA—acting through Rho kinase and perhaps other effectors—is very important for the contractile force on the uropod required for macrophage migration. Inhibition of rhoA or rho kinase leads to macrophages that have lengthy, extended uropods, and fail to migrate (Worthylake et al. 2001).

Recent work has emphasized that, in contrast to migration, integrin-mediated phagocytosis requires rho, but is independent of cdc42 and rac (Caron and Hall 1998). This is quite distinct from FcγR-mediated ingestion, which is dependent on cdc42 and rac, but independent of rho. The exclusive dependence of inte-

grin-mediated phagocytosis on rho may correlate with the morphology of this sort of ingestion. In FcγR-mediated phagocytosis, macrophage membrane protrudes around the target; in contrast, in integrin-mediated phagocytosis, target particles sink into the cytoplasm, and there is little or no protrusion of membrane around the particle, emphasizing the contractile rather than protrusive functions of cytoskeleton. This dependence on contractility is consistent with the requirement for rho guanosine triphosphate (GTP)ase, which seems most closely associated with this cytoskeletal function. Almost all studies of integrin-mediated phagocytosis have examined αMβ2; it remains to be determined whether the rho dependence of the process is true for integrins in general. There are many examples of macrophage phagocytosis via other integrins, including αVβ3, αVβ5, and α5β1.

There are other distinctions between the signals required for integrin-mediated spreading and phagocytosis. While syk is required for integrin β2-dependent cell spreading in macrophages (Vines et al. 2001), it is not necessary for αMβ2-mediated phagocytosis (Kiefer et al. 1998). Indeed, even quite broadly reactive tyrosine kinase inhibitors do not block αMβ2-mediated phagocytosis (Allen and Aderem 1996). Consistent with a different requirement of tyrosine kinases in integrin-dependent adhesion and phagocytosis, macrophages that fail to express the transmembrane tyrosine phosphatase CD45 show abnormal adhesion and spreading, but ingest normally (Roach et al. 1997). Together, these studies suggest that integrin ligation can activate a tyrosine kinase-initiated pathway in macrophages involving syk that likely leads to cdc42 and rac activation downstream from syk activation of PI 3-kinase. This pathway is required for events at the leading edge of an orientated macrophage, including lamellar protrusion, that are required for migration and spreading. In contrast, this pathway is not required for integrin-mediated ingestion. The data imply that there must be a pathway for rho activation during phagocytosis—independent of tyrosine kinases—that is sufficient to support integrin-mediated ingestion. Rho can be activated by certain G protein-coupled receptors, and there is evidence that integrin ligation can activate heterotrimeric G protein signaling (Erb et al. 2001; Wei et al. 2001). Thus, it is intriguing to speculate that integrin-mediated ingestion may be initiated or sustained by a pathway involving heterotrimeric G protein activation of rho. It is possible that protein kinase C, which also is required for integrin-mediated ingestion (Allen and Aderem 1995), synergizes with rho to activate phospholipase D (Du et al. 2000). Phospholipase D activity is closely correlated with integrin-mediated phagocytosis (Kusner et al. 1996; Serrander et al. 1996). Given these distinctions, it might be possible to target pharmacologically either integrin-mediated phagocytosis or integrin-mediated adhesion and migration without affecting the other function. This could be useful in modulating, e.g., presentation of particulate antigens, without interfering with other host defense functions of the macrophages.

6
Integrins as Components of Multimolecular Complexes on Macrophages

An emerging area of understanding is that integrins can function as components of multimolecular complexes at the plasma membrane that have unique functions that are more than the sum of the individual receptors. Although not the first to be discovered, complexes of growth factor receptors and integrins have received the most intense scrutiny because it is clear that these complexes are responsible for many of the survival or proliferative signals initially attributed to growth factor signaling in anchorage-dependent cells. For many years, it has been known that most nontransformed cells require adhesion for survival or proliferation, even in the presence of adequate growth factor. This role for adhesion is now clearly attributable to integrin signaling. While fibroblasts undergo only growth arrest on loss of adhesion, endothelial cells and epithelial cells die from a form of apoptosis termed anoikis ("homelessness"). Even fibroblasts require adhesion for survival in the absence of growth factors (Ilic et al. 1998). In fibroblasts, endothelial cells, and epithelial cells, complexes among integrins and EGF or platelet-derived growth factor (PDGF) receptors have been identified. The growth factor receptors in these complexes are phosphorylated to a greater extent and more efficiently signal microtubule-associated protein (MAP) kinase activation than receptors not associated with integrins (DeMali et al. 1999; Yu et al. 2000). In addition to survival or proliferation, these complexes may be important in cell migration in response to growth factors. Although these phenomena have not been studied extensively in macrophages, they may have relevance to macrophage biology. Colony-stimulating factor (CSF)-1 withdrawal leads to both cell rounding and apoptosis of murine macrophages (Pixley et al. 2001); both processes require the tyrosine phosphatase Src-homology-containing phosphatase (SHP)-1 (Roach et al. 1998; Berg et al. 1999), suggesting a possible functional link between the two phenomena. Moreover, deliberate inhibition of adhesion induces apoptosis in bone marrow-derived macrophages, suggesting that these cells may undergo anoikis. Finally, genetic defects that lead to diminished adhesion (e.g., absence of the transmembrane tyrosine phosphatase CD45 or the integrin CD18) lead to rapid apoptosis on growth factor withdrawal. These data are all consistent with an important role for cooperation between integrins and c-fms, the CSF-1 receptor, in macrophage biology. To date, no evidence about whether or not macrophage integrins form a complex with the CSF-1 receptor has been published.

A second multimolecular complex at the plasma membrane involves integrins and a family of proteins known as tetraspanins (Berditchevski 2001). As their name suggests, the tetraspanin family all have four transmembrane segments, with a large extracellular loop between the third and fourth segment and short aminoterminal and carboxyterminal cytoplasmic domains. There are at least 26 members of the tetraspanin family in higher eukaryotes, many of which have broad cellular distribution. There has been no systematic study of tetraspanin expression in macrophages, but several, including CD9 and CD81, which have

important integrin associations, are known to be expressed on macrophages. Tetraspanin–integrin complexes have been implicated both in cell adhesion and cell motility. Tetraspanins may contribute to these integrin-dependent phenomena by recruiting important signaling enzymes, such as phosphatidylinositol 4-kinase and protein kinase C, to sites of adhesion. Tetraspanins may also contribute to regulation of integrin expression at the plasma membrane or recycling from coated pits. Very little investigation has been done to date on the role of these undoubtedly physiologically important complexes in macrophage integrin function.

The association of specific integrins with particular proteases is important in cell migration through extracellular matrix, presumably because the interaction allows the adhesive integrin to direct the matrix degrading activity to specific sites on the cell surface. The association of matrix metalloproteinase (MMP)-2 with $\alpha V\beta 3$ has been shown to be involved in angiogenesis (Brooks et al. 1998), and the protease and integrin seem to act synergistically, since while intact collagen is not a ligand for $\alpha V\beta 3$, the integrin can recognize collagen that has been degraded by the MMP. Association of $\alpha V\beta 6$ with MMP-9 is implicated in metastasis of colorectal cancer (Niu et al. 1998), and $\alpha 2\beta 1$ ligation by collagen can induce synthesis of MMP-1 (Jones and Walker 1997). Although macrophage MMPs have been implicated in a variety of chronic inflammatory and destructive diseases, such as arthritis, emphysema, and atherosclerosis, there has not been a systematic examination of the significance of the MMP–integrin interaction for these diseases.

A second example of protease–integrin association involves a multimolecular complex involving integrins and the urokinase receptor (uPAR). UPAR is a glycan phosphoinositol (GPI)-linked receptor that has been coprecipitated with $\alpha V\beta 3$, $\alpha M\beta 2$, $\alpha 3\beta 1$, and $\alpha 5\beta 1$ in a variety of cells. Binding of the enzyme ligand plasminogen activator to uPAR enhances integrin-mediated adhesion and migration. The site of interaction of $\alpha M\beta 2$ with uPAR has been mapped to a region of the αM chain in the beta propeller domain. A peptide based on this αM sequence also inhibited uPAR association with $\alpha 3\beta 1$ and $\alpha 5\beta 1$, suggesting that a common site on uPAR interacts with diverse integrins. Other GPI-linked receptors can be physically associated with the $\alpha M\beta 2$ integrin, including FcγRIIIB and CD14 (Poo et al. 1995; Pfeiffer et al. 2001). FcγRIIIB function as a signaling or phagocytic receptor may depend on its association with $\alpha M\beta 2$. Finally, some transmembrane receptors (e.g., FcgRIIA; Petty and Todd 1996) physically associate with specific integrins; in these circumstances, integrins and associated receptors reciprocally influence each other's function.

The best-studied example of a plasma membrane multimolecular complex involving association of integrins with other adhesion receptors is the interaction of a subset of integrins with CD47 (Brown and Frazier 2001). CD47 is an Ig superfamily member that has five transmembrane segments that can be coprecipitated with $\beta 3$ integrins and $\alpha 2\beta 1$. It also may associate with other integrins, perhaps with affinity too low to be detected by coprecipitation. The known ligands for CD47 include thrombospondins, through which CD47 may participate in

recognition of the extracellular matrix, and signal regulatory protein (SIRP)α, a plasma membrane protein expressed on a variety of cells, through which CD47 may participate in cell–cell interactions. The unusual structure of CD47 suggests that it can act as a signaling molecule, and there is abundant experimental evidence that supports this hypothesis. In association with integrins, CD47 can activate heterotrimeric G proteins, especially Gi (Brown 2001); this complex may be important in the integrin-mediated functions that appear to depend on heterotrimeric G protein signaling. However, it is clear that not all signaling by CD47 is inhibited by pertussis toxin; in these cases, the proximal signals from CD47 ligation are uncertain. Ligation of the integrin-CD47 complex can lead to cell migration, activation, and aggregation, and mice lacking CD47 have a defect in integrin-mediated cell activation that leads to a deficiency in host defense. The significance of the complex for macrophage biology specifically has not been studied in detail.

7
Therapeutic Inhibition of Macrophage Integrin Function

The previous discussion makes it clear that there are a number of integrins expressed on macrophages that might be rational targets for therapeutic intervention, but several conceptual problems exist that may limit the utility of therapeutic integrin blockade. The first problem is that integrins present on macrophages are usually expressed on other cells as well. Therefore, integrin inhibition might lead to undesired side effects because of blockade of essential functions on these other cells. The second problem is redundancy in integrin function. For example, multiple β1 integrins recognize the extracellular matrix fibronectin, so that inhibition of cell binding to this ligand could require inhibition of as many as six different integrins. At the same time, too broad an inhibition of integrins could lead to unwanted side effects. For example, inhibition of β2 integrin function with monoclonal antibodies, although it has beneficial effects on acute survival from reperfusion injury (Winn et al. 1997), markedly increases susceptibility to bacterial infection (Mileski et al. 1993). Nonetheless, there are some diseases in which anti-integrin therapy has had clear utility. The greatest success to date has been the use of inhibitors of αIIbβ3, the major platelet integrin required for platelet aggregation, as acute anti-thrombotic therapy following angioplasty (Coller 2001).

There are several chronic inflammatory diseases that seem to be natural candidates for anti-macrophage integrin therapy because they are thought to play an important role in pathogenesis. These include multiple sclerosis, inflammatory bowel disease, various arthritides, and atherosclerosis. At this point, the data for involvement of integrins comes primarily from in vitro studies or experiments in animal models. Much of the work has focused on the integrin $\alpha4\beta1$ because of its predominant expression on leukocytes and its importance for transendothelial migration of both lymphocytes and macrophages. Thus, inhibition of $\alpha4\beta1$ has the possibility to block trafficking of these two cell types with-

out significant effect on other biologic processes. Moreover, since polymorphonuclear neutrophil (PMN) express little $\alpha 4\beta 1$, blockade of this integrin has less potential to increase susceptibility to infection than blockade of $\beta 2$ integrins, which are highly expressed on PMN. A humanized monoclonal antibody that blocks $\alpha 4\beta 1$ function is now in clinical trials for both multiple sclerosis and Crohn's disease. Other potentially important targets are the collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ that are expressed more widely than $\alpha 4\beta 1$. These two integrins are expressed on macrophages and on lymphocytes at sites of chronic inflammation, and inhibition of these integrins has a preventive or therapeutic effect in several murine models of chronic inflammation (de Fougerolles 2000). While most therapeutic effects of anti-$\alpha 4\beta 1$ may be attributable to effects on lymphocyte trafficking, inhibition of $\alpha 1$ and $\alpha 2$ blocks inflammation in diseases that do not have a lymphocyte component, implicating a therapeutic effect of blockade of these molecules on macrophages.

Because of the important role of macrophages in the pathogenesis of atherosclerosis and the early expression of VCAM-1 (an $\alpha 4\beta 1$ ligand) on endothelium overlying sites of intimal proliferation, atherosclerosis seems to be a good target for anti-integrin therapy (Li et al. 1993). Furthermore, ICAM-1 (a ligand for $\alpha L\beta 2$ and $\alpha M\beta 2$) likely contributes to the atherogenic process, which is modestly delayed in ICAM-1-deficient mice (Collins et al. 2000). While CD11b deficiency does not have the same protective effect against atherogenesis (Kubo et al. 2000), combined deficiency of $\alpha L\beta 2$ and $\alpha M\beta 2$ has not been investigated. Because $\alpha 4$ deficiency leads to embryonic lethality, atherosclerosis in $\alpha 4$-deficient mice has not been investigated. However, in some models, short-term treatment with anti-$\alpha 4$ antibodies or $\alpha 4\beta 1$ blocking peptides reduces both monocyte and lipid accumulation in plaques (Huo and Ley 2001), suggesting the possibility that successful targeting of monocyte $\alpha 4\beta 1$ could have a significant impact on generation of the atheromatous lesion.

Finally, a very promising target for anti-integrin therapy is in treatment of osteoporosis, a disease caused by increased bone resorption by osteoclast, a blood monocyte-derived cell, relative to bone formation by osteoblasts. The multinucleate osteoclast develops a specialized bone-apposed plasma membrane that is rich in the H^+ ATPase proton pump, allowing secretion of acid into a space between the cell and the mineralized surface of the bone, that is thought to be important in the process of bone resorption. The fact that the pH of this compartment is quite different from the surrounding medium implies that there must be a tight barrier between this compartment and the rest of the milieu of the osteoclast. It is thought that integrin $\alpha V\beta 3$-mediated adhesion is required for this tight seal. During differentiation from monocyte to osteoclasts, cells begin to express the integrin $\alpha V\beta 3$ at high levels. Moreover, $\alpha V\beta 3$ is present at the circumference of the acid bone-resorbing compartment. In mice, $\beta 3$ deficiency leads to enhanced bone mineralization (osteosclerosis) (McHugh et al. 2000), and inhibitors of $\alpha V\beta 3$ significantly block bone resorption in several animal models of osteoporosis (Crippes et al. 1996; Engleman et al. 1997). Blockade of $\alpha v\beta 3$ also inhibits tumor-induced angiogenesis and neovascularization at in-

flammatory sites (Eliceiri and Cheresh 1999). While this anti-angiogenic effect is a potential problem, these antagonists apparently have no effect on already established vasculature. Indeed, the additional effect of antagonizing $\alpha V\beta 3$ may make this integrin a target in inflammation and cancer as well as osteoporosis.

Because of the side effects associated with general inhibition of $\beta 2$ integrins, these have been considered poor targets for therapeutic intervention. However, as we learn more about the signaling cascades associated with integrin ligation, it may be possible to develop more specific inhibitors that can block some aspects of integrin signaling without disturbing integrin-mediated adhesion and migration. Such a strategy might lead to blockade of consequences of chronic adhesion—for example, macrophage synthesis of growth factors and chemoattractants for smooth muscle cells at atherosclerotic plaques—while allowing normal migration of PMN and macrophages to sites of acute infection. However, neither the molecular mechanisms required for activation of adhesion and migration—"inside out signaling"—or the signaling pathways impacted by integrin engagement—"outside in signaling"—are known in sufficient detail to exploit this strategy for therapeutic purposes. This both justifies further basic research into understanding the molecular mechanisms of regulation of integrin function and holds hope that the future will see important advances in manipulation of integrin targets for therapeutic purposes.

8 References

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Macrophage Targets in Inflammation: Purinergic Receptors

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Abstract Extracellular adenosine and adenine nucleotides are potent endogenous modulators of inflammation that accumulate in the pericellular space under various physiological and pathological conditions. Their action is mediated by plasma membrane receptors named P1 (adenosine) or P2 (adenine nucleotides). Adenosine is generated extracellularly by ubiquitous ecto-ATPases/ecto-nucleotidases that dephosphorylate adenosine 5′-triphosphate (ATP). Adenosine is then either finally degraded by adenosine deaminase or taken up by the cells. Activation of P1 receptors by adenosine mainly inhibits, and activation of P2 receptors by adenine nucleotides mainly stimulates inflammatory cell responses. Differential expression by inflammatory cells and modulation during differentiation make P1 and P2 receptors potential targets for development of novel anti-inflammatory drugs.

Keywords Adenosine, Adenine, Cytokines, Ecto-ATPases, Adenosine 5′-triphosphate, Intracellular parasites

1 Introduction

It has been known for some years that adenosine inhibits in vitro inflammatory cell responses, and there are grounds to believe that locally released adenosine downmodulates inflammation in vivo by acting at A_{2A} receptors and may mediate the in vivo therapeutical effect of anti-inflammatory drugs (Cronstein et al. 1983; Cronstein et al. 1993; Ohta and Sitkovsky 2001). It was less appreciated until recently that adenosine precursors, the adenine nucleotides, may also modulate responses of inflammatory cells, among which, most notably, are the macrophages. At inflammatory sites, cell injury or activation causes release of substantial amounts of intracellular nucleotides that may dramatically affect responses of resident or infiltrating inflammatory cells (Di Virgilio 1995; Morabito et al. 1998) (the cytoplasmic ATP concentration ranges from 5 to 10 mM in most cell types, while that of other nucleotides, e.g., uridine triphosphate (UTP), can reach about one third of that of ATP) (Lazarowski and Boucher 2001). Extracellular nucleotides trigger a complex network of autocrine/paracrine interactions that, depending on the relative amounts of nucleotide or nucleoside present, the type and level of specific plasma membrane receptors expressed, and the state of priming of neighbouring cells by other inflammatory mediators, will eventually lead to the amplification or inhibition of the inflammatory response. In addition, the ubiquitous ecto-ATPases/ecto-nucleotidases, that rapidly hydrolyse extracellular nucleotides to adenosine, are powerful modulatory elements. Adenosine is then either finally degraded by adenosine deaminase (ADA), or taken up by the cells to be further utilized in intracellular synthetic pathways. While a number of agents able to modulate the effects of adenosine have been available for several years and are currently tested for clinical use, drugs that interfere with inflammatory cell stimulation by extracellular nucleotides have attracted interest only very recently, and none of them has entered clinical trials.

2 Purinergic Receptors: What Are They?

The name "purinergic" receptors was initially coined on a purely functional and pharmacological basis, indicating those plasma membrane receptors mediating the action of extracellular adenine nucleotides or nucleosides, i.e. ATP or adenosine. As it has recently become clear that some P2 receptor subtypes are highly specific for UTP or uridine diphosphate (UDP), it has been agreed to drop the word "purinergic" (UTP and UDP are pyrimidines) and simply name receptors for extracellular nucleotides or nucleosides as P2 or P1, respectively. The original classification based on the pharmacological profile has been replaced by that based on the molecular structure; accordingly, P2 receptors are grouped into two subfamilies (P2Y and P2X), numbering eight and seven members, respectively, and P1 receptors into four subtypes. Very recently, the receptor for uri-

Table 1 Classification of mammalian adenosine/P1 receptors

Subtype	Amino acid number	Signal transduction	
A_1	326–328	$\uparrow IP_3$	$\downarrow$cAMP
A_{2A}	409–411		$\uparrow$cAMP
A_{2B}	328–332	$\uparrow IP_3$	$\uparrow$cAMP
A_3	318–320	$\uparrow IP_3$	$\downarrow$cAMP

Size of P1 receptororthologues depends on the speciesof origin.

Table 2 Classification of mammalian P2Y and P2X receptors

Subtype	Amino acid number	Preferred naturally occurring agonist	Signal transduction	
$P2Y_1$	362	ADP	$\uparrow IP_3$	$\downarrow$cAMP
$P2Y_2$	373	UTP, ATP	$\uparrow IP_3$	
$P2Y_4$	352	UTP	$\uparrow IP_3$	
$P2Y_6$	379	UDP	$\uparrow IP_3$	
$P2Y_{11}$	371	ATP	$\uparrow IP_3$	$\uparrow$cAMP
$P2Y_{12}$	342	ADP		$\downarrow$cAMP
$P2Y_{13}$	334	ADP	$\uparrow IP_3$	$\uparrow\downarrow$cAMP
PSY_{14}	338	UDP-glucose	$\uparrow IP_3$	
$P2X_1$	399	ATP	Ion currents	
$P2X_2$ [a]	472	ATP	Ion currents	
$P2X_3$	397	ATP	Ion currents	
$P2X_4$ [a]	388	ATP	Ion currents	
$P2X_5$	455	ATP	Ion currents	
$P2X_6$	379	ATP	Ion currents	
$P2X_7$	595	ATP	Ion currents Protein–protein interaction	
$P2X_2/P2X_3$		ATP	Ion currents	
$P2X_1/P2X_5$		ATP	Ion currents	
$P2X_4/P2X_6$		ATP	Ion currents	

[a] Splice variantsof $P2X_2$ and $P2X_4$ have been identified. P2X receptors may assemble as heteroligomers. At $P2Y_1$ and $P2Y_{12}$ ATP may act as an antagonist. $P2Y_{13}$ mediatesinhibition of adenylatecyclase at low and stimulation at high concentrations.

dine 5′-diphosphoglucose (UDP-glucose) has been included in the P2 family (Tables 1 and 2).

2.1 P2 Receptors

P2 receptors are divided into two subfamilies: P2X (ligand-gated ion channels, ionotropic, receptors) and P2Y (G protein-coupled, metabotropic, receptors). P2X receptors are activated by extracellular ATP (the only known physiological ligand), and mediate fast mono- and di-valent cation (Na^+, K^+ and Ca^{2+}) fluxes across the plasma membrane (North 2002). Intriguingly, permeability of some

P2X receptors (especially $P2X_7$, but to a lesser extent also $P2X_2$ and $P2X_4$) increases during continuous ATP stimulation or upon application of repeated ATP pulses (Di Virgilio 1995; Falzoni et al. 1995; Virginio et al. 1999; Khakh et al. 1999). The molecular basis of this odd phenomenon is as yet unknown, but it is hypothesized that it might depend on the recruitment of additional receptor subunits, thus increasing the size of the channel pore. All P2X receptors are multimeric structures of which seven basic subunits, and some splice variants, have been cloned ($P2X_{1-7}$).

An open problem is P2X receptor subunit stoichiometry and composition. Growing consensus supports the view that P2X receptors assemble as trimers or hexamers (Nicke et al. 1998), but assembly into tetramers has also been reported (Kim et al. 1997). Functional expression studies show that heterologously expressed P2X subunits may assemble to form heteromultimeric structures, and patch-clamp data suggests that native P2X receptors may also assemble as heteromultimers (Lewis et al. 1995; Torres et al. 1999). A relevant exception is $P2X_7$, the P2X receptor mainly expressed by macrophages. While there is good evidence to support a tri/hexameric stoichiometry for this receptor, there is likewise strong evidence that the $P2X_7$ subunit does not assemble with any other P2X subunit (Torres et al. 1999; Kim et al. 2001b). Size of P2X subunits is comprised between 379 ($P2X_6$) and 595 ($P2X_7$) amino acids, for a predicted molecular weight of 42 to 65 kDa. Hydropathy plots and absence of a leader sequence predict a membrane topology with only two transmembrane stretches separated by a bulky extracellular region with both the N- and C-termini on the cytoplasmic side (Brake et al. 1994; Surprenant et al. 1996). The extracellular domain contains 10 cysteines and 2–6 N-linked glycosylation sites.

Within the P2X subfamily, a special place is occupied by $P2X_7$ for several reasons: (1) it has a long (242 residues) cytoplasmic carboxy-terminal tail compared to the other members of the family; (2) the COOH tail contains a hydrophobic stretch that might insert into the plasma membrane or interact with cytoplasmic vesicles; (3) it does not heteropolymerize with other P2X subunits; (4) its activation needs ATP concentrations that are 10- to 100-fold higher than those required to activate other P2X receptors; (5) it is the P2X receptor that more readily undergoes a large increment in conductance (channel-to-pore transition) upon sustained stimulation with ATP (in fact it is the only P2X receptor for which this phenomenon has been reproducibly demonstrated and extensively characterized); (6) it is mainly, although not exclusively, localized to immune cells; (7) it is the only P2X receptor whose activation undisputedly triggers cell death.

Of particular relevance for the participation of $P2X_7$ in the modulation of macrophage responses is the finding that its COOH tail harbours an amino acid stretch (573–590) highly homologous (about 90%) with the lipopolysaccharide (LPS)-binding site of LPS-binding protein (Denlinger et al. 2001). Additional studies have provided evidence that $P2X_7$ interacts with several extracellular, transmembrane or cytoskeletal proteins to form a highly complex and versatile signalling complex (receptosome) (Denlinger et al. 2001; Kim et al. 2001a). At

least eleven $P2X_7$-associated proteins have been conclusively identified: laminin $\alpha 3$, integrin $\beta 2$, receptor protein tyrosine phosphatase β (RPTPβ), α-actinin, β-actin, supervillin, heat shock protein (Hsp) 90, heat shock cognate (Hsc) protein 71, Hsp70, phosphatidylinositol 4-kinase (PI4 K) 230 and membrane-associated guanylate kinase (MAGuK) P55 (Kim et al. 2001a). Finally, all P2X receptors contain in the N-terminus a consensus sequence for protein kinase C (Thr-X-Lys/Arg).

P2Y receptors are members of the rhodopsin-like G protein superfamily with seven transmembrane domains (North and Barnard 1997; von Kugelgen and Wetter 2000). They number from 333 ($P2Y_{13}$) to 379 ($P2Y_6$) amino acids, for a predicted molecular mass of 36–42 kDa. Agonist selectivity is a discriminant feature of P2Y receptors: $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ are selective for adenine nucleotides, whereas other members of the P2Y family can also be activated by uracil nucleotides. Furthermore, $P2Y_1$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ are selectively activated by nucleoside diphosphates, while the other P2Y receptors are preferentially activated by nucleoside triphosphates. All P2Y receptors, with the exception of $P2Y_{12}$, couple to phospholipase C, thus causing inositol triphosphate formation and Ca^{2+} release from intracellular stores. Coupling to phospholipase D has also been described (Purkiss and Boarder 1992). $P2Y_1$ and $P2Y_2$ mediate inhibition of adenylate cyclase, while $P2Y_{11}$ mediates stimulation. $P2Y_{13}$ mediates inhibition of adenylate cyclase at low and stimulation at high concentration. The receptor for UDP-glucose has been included in the P2Y family as $P2Y_{14}$. This receptor, expressed in a wide variety of human tissues, couples to Ca^{2+} release from intracellular stores (Chambers et al. 2000).

2.2 P1 Receptors

P1 receptors are defined as those plasma membrane receptors that bind adenosine. This family comprises four members: A_1, A_{2A}, A_{2B} and A_3. All adenosine receptors are seven membrane-spanning and couple via G proteins to phospholipase C and/or adenylate cyclase. Activation of the A_1 subtype causes phospholipase C stimulation and a decrease in cyclic adenosine monophosphate (cAMP), while activation of A_{2A} causes only stimulation of adenylate cyclase. Activation of A_{2B} causes an elevation of both IP_3 and cAMP. Finally, activation of A3 causes a decrease in cAMP and an increase in IP_3 concentration (Ralevic and Burnstock 1998). Like all the other members of the serpentine receptors superfamily, P1 receptors were until recently thought of as monomeric molecules, but a recent paper by Yoshioka et al. (2001) suggests that A_1 and $P2Y_1$ receptors may form heteromeric structures with $P2Y_1$-like agonistic pharmacology.

3
Sources and Mechanism for ATP Release into the Extracellular Space

There is no doubt that ATP is a major neurotransmitter in the central and peripheral nervous system, often in combination with other mediators. Less appreciated is the occurrence of ATP release outside the nervous system, but proof that this is a much more frequent event than commonly thought is being provided by an increasing number of laboratories (Dubyak et al. 2002; Schwiebert et al. 2002). Concentration of ATP in the blood or in the interstitial fluid under resting conditions is thought to be in the low nanomolar range, although this is likely to be a large underestimate of the actual ATP concentration in the pericellular space. Most cell types have been shown to continuously release ATP thus generating an autocrine tonic stimulation of P2 receptor (Ostrom et al. 2000), that may even lead to P2-receptor desensitisation. Several basal cellular parameters (e.g. the resting cytoplasmic Ca^{2+} and cAMP concentration, and the basal arachidonic acid release) appear to be modulated by the tonic stimulation of P2 receptors (Grierson et al. 1995; Ostrom et al. 2000). More interestingly, several agents are capable of inducing ATP release thus raising several fold the concentration of this nucleotide in the proximity of the plasma membrane (Ferrari et al. 1997b; Sperlagh et al. 1998; Warny et al. 2001).

One of the most important sources of ATP release are platelets. These cells store ATP up to a several millimolar concentration within their dense granules, thus massive ATP release, up to 20–50 μM, occurs during platelet aggregation (Beigi et al. 1999). Since activated platelets adhere to leukocytes and can establish protected compartments at the site of interaction with adjacent cells, it has to be expected that much higher ATP concentrations can be reached within these secluded environments, in a way reminiscent of the high concentrations achieved by neurotransmitters secreted into the synaptic cleft. Endothelial cells are another important source of extracellular ATP in response to shear stress (Bodin et al. 1991), swelling (Oike et al. 2000), or stimulation of plasma membrane receptors (Yang et al. 1994). Furthermore, leukocytes stimulated with bacterial endotoxin (LPS) also release ATP (Ferrari et al. 1997b; Sperlagh et al. 1998; Sikora et al. 1999; Imai et al. 2000; Warny et al. 2001; but see also Grahames et al. 1999 and Beigi and Dubyak 2000). There is hint that some well-known anti-inflammatory drugs (e.g. sulphasalazine and methotrexate) owe their action to their ability to promote ATP release from macrophages and conversion of this nucleotide to adenosine (Morabito et al. 1998).

While it is obvious that cell lysis may well be responsible for the discharge of cellular ATP, how this nucleotide is extruded from intact cells is still an open question. As mentioned above, platelets and a few other cell types certainly release ATP by exocytosis of their cytoplasmic granules (Meyers et al. 1982; Lages and Weiss 1999), but secretion of ATP from many other different cell types does not appear to occur via stimulated exocytosis. It has been suggested that ATP might be stored within vesicles originating from the Golgi and released by constitutive exocytosis (Maroto and Hamill 2001). Plasma membrane transporters

such as the multidrug resistance protein (Abraham et al. 1993) or the cystic fibrosis protein (CFTR) (Jiang et al. 1998) have also been candidated to the role of ATP transporters. Connexin 43 hemichannels have been reported to mediate ATP release (Cotrina et al. 1998), but this evidence has been recently questioned (Romanello et al. 2001). Rather intriguingly, it has been shown that transfection with the differentiation antigen CD39 (which is a member of plasma membrane ecto-nucleoside triphosphate diphosphohydrolase family) enhances ATP transport across the plasma membrane of *Xenopus* oocytes (Bodas et al. 2000). Finally, data by Baricordi et al. (1999) show that transduction of human lymphoblastoid cell lines with the $P2X_7$ receptor increases their ability to release ATP, thus suggesting that $P2X_7$ might participate in ATP secretion.

4 Systems Involved in Degradation of Extracellular ATP

Once in the extracellular space, ATP is quickly degraded by very active ecto-enzymes grouped into four families: ecto-nucleotide triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatase and ecto-5′-nucleotidase (Zimmermann 2000, 2001). E-NTPDases cleave ATP, adenosine diphosphate (ADP) and several other purine and pyrimidine nucleotides to AMP and Pi. An important member of this family is the lymphocyte differentiation marker CD39. E-NPPases hydrolyse ATP to AMP and Ppi, ADP to AMP and Pi or NAD^+ to nicotinamide mononucleotide. E-NPPases are known as differentiation antigens for plasma cells, motility-stimulating proteins (autotaxins) or neural differentiation and tumour surface markers. Alkaline phosphatases are non-specific ecto-phosphomonoesterases with a broad substrate specificity. Finally, ecto-5′-nucleotidases catalyse the conversion of nucleoside 5′-monophosphates to the respective nucleoside and Pi. Ecto-5′-nucleotidase is the main enzyme responsible for the extracellular generation of adenosine.

5 P1 and P2 Receptor Subtypes Expressed by Macrophages

An extensive characterization of P1 receptor expression by macrophages has not yet been carried out. However, broadly converging biochemical and pharmacological data from human monocyte/macrophages or mouse macrophages and human and mouse macrophage cell lines suggest that all the four P1 subtypes are expressed by these cells (McWhinney et al. 1996; Xaus et al. 1999; Montesinos and Cronstein 2001; Johnston et al. 2001). As regards P2 receptors, mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ has been amplified from monocytes and macrophages (Di Virgilio et al. 2001a; Dubyak 2001). Expression of $P2Y_{11}$ has been demonstrated in human dendritic cells (Wilkin et al. 2001), but as yet there is no published evidence that it may also be present in macrophages. Expression of members of the P2X subfamily is much less characterized, with the exception

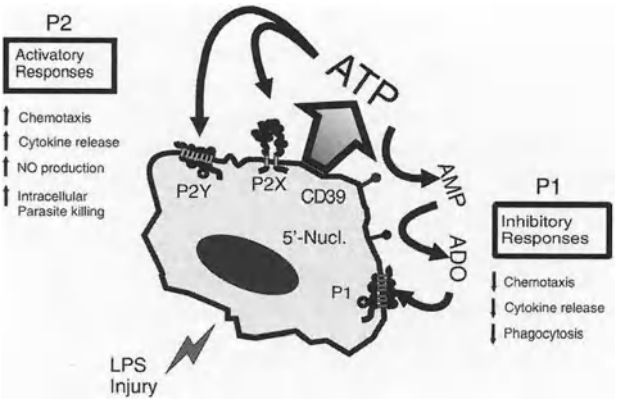

Fig. 1 Autocrine/paracrine loop based on ATP release from the macrophage and P2/P1 receptor activation

of $P2X_7$, that has been the focus of intense investigation over recent years (Di Virgilio et al. 2001a,b). While there is no doubt that $P2X_7$ is the main P2X receptor subtype expressed by macrophages (and very likely also by monocytes, despite previous evidence of the contrary) (Falzoni et al. 2000; Gu et al. 2000; Gudipaty et al. 2001), scattered evidence suggests that $P2X_1$ and $P2X_4$ might also be expressed, at least at certain developmental stages (Buell et al. 1996; Soto et al. 1996).

6 Role of P1 and P2 Receptors in Macrophage Physiology

While it is generally appreciated that extracellular ATP has an important role as a neuromediator, the notion that this nucleotide may also be a mediator of inflammation has received attention only very recently. Macrophages are fully equipped to exploit the potential of nucleotides and nucleosides as extracellular messengers as (1) they express P2Y, P2X and P1 receptors, (2) express ecto-ATPase/nucleotidase that convert nucleotides to nucleosides, and (3) can release ATP. Thus, macrophages are not only the target of ATP released by neighbour cells, but also the centre of an ATP-based autocrine/paracrine loop (Fig. 1). However, our understanding of signalling via extracellular nucleotides in the immune system is still very rudimentary. Some authors have suggested that extracellular nucleotides might be early signals for alerting the immune system of an impendent danger, and thus drive recruitment and activation of inflammatory cells (Greenberg et al. 1988; Di Virgilio et al. 1995; Gallucci and Matzinger 2001). Nucleotides have a strong macrophage chemotactic activity (McCloskey et al. 1999; Oshimi et al. 1999), activate the NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase, especially in the presence of phagocytic particles (Schmid-Antomarchi et al. 1997), stimulate release of interleukin (IL)-1α, IL-1β, IL-8 and IL-18 (Perregaux and Gabel 1994; Ferrari et al. 1997a; Perre-

gaux and Gabel 1998; Perregaux et al. 2000; Warny et al. 2001), drive the externalisation of active caspase-1 (Laliberte et al. 1999), trigger nitric oxide generation (Denlinger et al. 1996), increase expression of tumour necrosis factor (TNF)-α mRNA (Tonetti et al. 1995), and finally ATP at high doses has a potent cytotoxic effect (Steinberg and Silverstein 1987; Murgia et al. 1992). The ATP-dependent cytotoxic activity is of particular interest because a side-effect, not shared by other cytotoxic stimuli, is the killing of intracellular parasites (i.e. *Mycobacterium tuberculosis*) that are normally able to survive within phagocytic vacuoles. A number of different laboratories have shown that stimulation with ATP of macrophages that have ingested *Mycobacteria* causes an increased rate of phagosome-lysosome fusion that greatly enhances the microbicidal efficacy of the phagocytes (Molloy et al. 1994; Lammas et al. 1997; Fairbairn et al. 2001; Mancino et al. 2001). In the context of granulomatous diseases such as tuberculosis or sarcoidosis, it is of interest that one of the P2X subtypes, $P2X_7$, appears to be necessary for the formation of multinucleated giant cells (MGCs) (Chiozzi et al. 1997; Falzoni et al. 2000). A recent report shows that this same receptor is overexpressed in macrophages from sarcoidosis patients (Mizuno et al. 2001). The $P2X_7$ receptor has also an important role in osteoclast differentiation and in communication between osteoblasts and osteoclasts (Morrison et al. 1998; Jorgensen et al. 2002).

Most attention has so far concentrated on the involvement of $P2X_7$ in inflammation; however, other P2 receptor are increasingly implicated in macrophage responses to nucleotides. $P2Y_1$ and/or $P2Y_2$ are likely to be the main receptors mediating monocyte/macrophage chemotaxis in response to nucleotides, while $P2Y_6$ is coupled to release of the granulocyte selective chemokine IL-8. An intriguing, and as yet little studied subtype is $P2Y_{11}$. This receptor appears to have an important role in the maturation of dendritic cells, alone or in association with other P2 receptors (Wilkin et al. 2001; La Sala et al. 2001). Whether it is also involved in monocyte/macrophage differentiation is not yet known.

Participation of P1 and P2 receptors in the modulation of so many different macrophage responses makes these molecules obvious targets for the development of innovative anti-inflammatory drugs. The clinical use of P1 or P2 agonists/antagonists is complicated by the several side-effects due to the widespread expression of their receptors in many different organs. Thus, one should either concentrate on the development of agents able to affect the local nucleoside or nucleotide concentration, or try to exploit the tissue-specific expression of P1and P2 subtypes. A simpleminded, but probably not as naïve as it might appear, approach would be the attempt to modulate the local concentration of the P1 and/or P2 agonists, i.e. adenosine and the nucleosides di- and tri-phosphates (ATP, ADP, UTP and UDP). This strategy is supported by two observations: (1) the mechanism of action of some anti-inflammatory drugs is understood to be mediated by the local accumulation of adenosine (Montesinos et al. 1998), and (2) an excessive accumulation of extracellular ATP as a consequence of CD39 (ecto-ATPase) reduced activity severely impairs inflammatory cell mi-

gration (Goepfert et al. 2001). These observations make CD39 an additional appealing target for pharmacological modulation of inflammation.

Design and synthesis of selective P1 agonists or antagonists has been the field of extensive investigation over recent years, and some compounds are being evaluated for clinical use (see Baraldi and Borea 2000, and Linden 2001 for recent reviews). In contrast, comparatively fewer agonist or antagonists selective for P2Y or P2X subtypes are available. At the moment, $P2Y_{12}$ is the only receptor targeted by antagonists widely used in clinical trials (Storey 2001). But $P2Y_{12}$ is not expressed by macrophages. An interesting selective antagonist of $P2Y_1$ (MRS 2179) has been recently described, but no clinical data are as yet available (Camaioni et al. 1998). Stable agonists for the $P2Y_2$ receptor are available for the local treatment of respiratory and ophthalmic diseases (cystic fibrosis, dry eye syndrome) (Boeynaems et al. 2001). Some stable ATP analogues (α, β-methylene ATP and β,γ-methylene ATP) have a strong preference for P2X over P2Y receptors (Ralevic and Burnstock 1998) and might be used to selectively stimulate P2X responses. A few selective antagonists are available for P2X receptors. The most widely used is pyridoxalphosphate-6-azophenyl 2′-4′-disulfonic acid (PPADS), a pyridoxal phosphate derivative that, however, may also inhibit $P2Y_1$. PPADS is a non-competitive antagonist that is thought to form Schiff's bases with lysines (possibly K64 and K311) that are key constituents of the ATP binding site (Thompson 2002). The same mechanism of action is shared by another irreversible blocker: oxidized ATP. This compound too, thanks to its aldehyde groups, covalently binds the same lysines and irreversibly blocks P2X receptors (Murgia et al. 1993). Oxidized ATP was initially thought to be selective for $P2X_7$, but it is now clear that it blocks other P2X subtypes. Another compound, the quinoline derivative 1-[*N,O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-l-tyrosyl]-4-phenylpiperazine (KN-62) originally introduced as a P2 reagent by Gargett and Wiley (1997), is proving very useful as a lead compound for the synthesis of novel $P2X_7$ blockers (Baraldi et al. 2000; Ravi et al. 2001). As of now, none of these compounds has entered clinical trials.

7 Conclusions

Receptors for extracellular nucleotides and nucleosides are emerging as novel and important modulators of inflammation. Expression of specific P1 and P2 subtypes by different white blood cell types at various developmental stages confers to these receptors an intriguing plasticity that on the one hand underlines their importance in leukocyte function and differentiation, and on the other suggests new avenues for the development of innovative anti-inflammatory drugs. The relevance of P2 receptors in the overall inflammatory process is further underscored by their increasingly recognized role in nociception (Burnstock 2000; Cook and McCleskey 2002). These studies suggest that modulation of signal transduction based on extracellular ATP and its metabolites may allow us to design a new generation of drugs targeted at many manifestations of

inflammation, ranging from pain sensation to leukocyte recruitment and pathogen killing.

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Macrophage Lipid Uptake and Foam Cell Formation

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Abstract The attraction and sub-endothelial migration of monocytes or macrophages (Mϕ) to the early atherosclerotic lesion or fatty streak are central events in atherogenesis. During migration through the endothelium these cells differentiate to Mϕ, which subsequently become activated in the milieu of cytokines and reactive products secreted by the dysfunctional endothelial layer, underlying smooth muscle cells and resident tissue leukocytes. As the lesion advances, the products secreted by activated Mϕ further escalate the inflammatory pro-

cess, increasing the recruitment of more activated inflammatory cells. Mϕ within the lesion also actively accumulate lipid to become foam cells. Therefore, interventions that target the escalation of the inflammatory cascade, foam cell differentiation, and foam cell lipid metabolism should have distinct therapeutic benefits in altering the progression of CAD. In this chapter, various aspects of Mϕ lipid metabolism and foam cell formation are explored. These include the sources of cholesterol for foam cell formation, receptor-dependent and receptor-independent uptake pathways by which foam cells may accumulate cholesterol, cholesterol trafficking within Mϕ, cholesterol efflux from Mϕ, and foam cell death. All of these processes represent possible therapeutic targets in CAD.

Keywords CD36, Cholesterol efflux, Foam cells, Macrophages, Macrosialin, Scavenger receptors, SR-A, SR-BI

Abbreviations

ABCA1	ATP-binding cassette, subfamily A, member 1
ACAT	Acyl-CoA:cholesterol acyltransferase
AcLDL	Acetylated low-density lipoprotein
aP2	Fatty acid-binding protein
β-VLDL	β-Very low-density lipoprotein
CAD	Coronary artery disease
CE	Cholesteryl ester
FC	Free cholesterol
HDL	High-density lipoprotein
LXR	Liver X receptor
Mϕ	Macrophage(s)
oxLDL	Oxidized low-density lipoprotein
PPAR	Peroxisome proliferator-activated receptor
SR-A	Scavenger receptor class A
SR-BI	Scavenger receptor class B, type I
RXR	Retinoid X receptor
VLDL	Very low-density lipoprotein

1 Introduction

The attraction and sub-endothelial migration of monocytes or macrophages (Mϕ) to the early atherosclerotic lesion or fatty streak are central events in atherogenesis (Ross 1999; Glass and Witztum 2001). One of the earliest observed endothelial changes is the surface arrest of monocytes. During migration through the endothelium these cells differentiate to Mϕ, which subsequently become activated in the milieu of cytokines and reactive products secreted by the dysfunctional endothelial layer, underlying smooth muscle cells and resident tis-

sue leukocytes. As the lesion advances, the products secreted by activated Mϕ further escalate the inflammatory process, increasing the recruitment of more activated inflammatory cells. Mϕ within the lesion also actively accumulate lipid to become foam cells. Therefore, interventions that target the escalation of the inflammatory cascade, foam cell differentiation and foam cell lipid metabolism should have distinct therapeutic benefits in altering the progression of CAD. In this chapter the various aspects of Mϕ lipid metabolism and foam cell formation that may represent therapeutic targets are explored.

2
Cholesterol Sources and Foam Cell Development

Two types of lipid oils, cholesteryl ester and triglyceride, can produce cellular lipid droplet accumulation and cause a foamy appearance in tissue sections prepared with organic solvents. Lipid droplets in tissues of cells prepared without extracting solvents can be stained with lipid-soluble dyes such as oil red O. Chemical studies of human atherosclerotic lesions show that the major lipid oil accumulating in lesions is cholesteryl ester (CE) and not triglyceride (Garner et al. 1997).

Exogenous cholesterol, not endogenously synthesized cholesterol, is the major source of cholesterol for foam cell formation. De novo synthesis of cholesterol functions to maintain cellular cholesterol levels in the absence of an exogenous source, and key enzymes in the cholesterol biosynthetic pathway are downregulated when an exogenous source of cholesterol is present (Goldstein and Brown 1990). Plasma lipoproteins are the major source of the exogenous cholesterol of foam cells, although other sources, such as cholesterol-rich platelet fragments (Curtiss et al. 1987), cause cholesterol loading in cultured Mϕ. Lipoproteins form emulsions of particles of varying densities that consist of a hydrophobic core containing neutral lipids, CE and triglycerides surrounded by an outer phospholipid monolayer containing a small amount of free cholesterol (FC) and protein (apolipoprotein). The bulk of the cholesterol (e.g., 80% in LDL) is carried as CE in the core of the particle.

2.1
Modified Forms of LDL

Although fasting plasma levels of LDL are strongly correlated with the extent of atherosclerosis, native plasma LDL is a poor inducer of foam cell formation in cultured Mϕ (Brown and Goldstein 1983; Steinberg et al. 1989). This finding can be explained by the combination of downregulation of LDL receptors (Brown and Goldstein 1983) and, as described below, specific characteristics of LDL-cholesterol metabolism in Mϕ (Tabas et al. 1990). A possible resolution to the apparent paradox of epidemiological data linking LDL to atherogenesis and foam cell formation is that LDL is modified to an "atherogenic" form in the subendothelium (Steinberg 1995). In an in vitro model to explore this concept, LDL modified by oxidation or acetylation has been widely studied. Native LDL is rec-

ognized by the LDL receptor by virtue of specific receptor-binding domains of the apolipoprotein B-100 moiety of LDL (Brown and Goldstein 1986). In contrast, AcLDL, due to acetylation of key lysine residues on apolipoprotein B-100, is not recognized by the LDL receptor but rather by another class of receptors, the so-called scavenger receptors (Goldstein et al. 1979; Krieger and Herz 1994). Scavenger receptor class A (SR-A) is widely expressed on differentiated Mϕ and mediates the constitutive uptake of AcLDL, leading to massive CE accumulation in most cultured Mϕ models (Brown and Goldstein 1983; Krieger and Herz 1994). Although acetylation of LDL does not occur in vivo, oxidation of LDL is a physiologically plausible process that also renders LDL a ligand for scavenger receptors and other cell-surface scavenger receptors that are not downregulated by cellular cholesterol loading (Steinberg 1997). Immunohistochemical studies have documented the presence of oxLDL in atherosclerotic lesions, and a wide variety of cell-culture studies have demonstrated potentially atherogenic effects of oxLDL, such as induction of atherogenic endothelial cell responses and smooth muscle cell proliferation (Steinberg 1997). Further support for the oxidation hypothesis includes recent data showing that deletion of the Mϕ-specific enzyme, 12/15-lipoxygenase, which is involved in the synthesis of oxygenated fatty acids, reduces levels of atherosclerosis in *ApoE* $^{-/-}$ mice (Cyrus et al. 1999).

A widely held misconception, however, is that LDL oxidized by most standard in vitro methods induces foam cell formation (i.e., marked CE accumulation) in cultured Mϕ. In fact, quite a few studies have shown that while Mϕ incubated with oxLDL internalize lipoprotein extensively, the cells accumulate mostly FC (Roma et al. 1990; Ryu et al. 1995; Klinkner et al. 1995; Musanti and Ghiselli 1993). The mechanism may be related to reduced CE content of the lipoprotein as a result of oxidation (Ryu et al. 1995) or to incomplete lysosomal degradation of oxLDL components, which in turn may lead to decreased export of FC from lysosomes (Lougheed et al. 1991; Hoppe et al. 1994; Maor et al. 1995). Additional in vitro modifications of oxLDL, such as by FC enrichment (Greenspan et al. 1997), are able to convert this lipoprotein into one that can cause substantial CE accumulation in cultured Mϕ, but it is still not certain whether these additional modifications of oxLDL occur in vivo. Recently, investigators have shown that SR-A-deficient and CD36-deficient mice have reduced, but by no means absent, foam cell lesions (Suzuki et al. 1997; Febbraio et al. 2000). Whereas proponents of the oxidation hypothesis might argue that the presence of foam cells in these knockout mice implicates a role for other oxLDL receptors in foam cell formation in vivo, an equally plausible interpretation is that a significant portion of foam cell formation involves other types of atherogenic lipoproteins altogether.

2.2 Aggregated Lipoproteins

Aggregated lipoproteins, which are not necessarily recognized by scavenger receptors, have been demonstrated in both early and late atherosclerotic lesions and are very potent inducers of foam cell formation in cultured Mϕ (Khoo et al.

1988; Suits et al. 1989; Hoff et al. 1990; Xu and Tabas 1991). Freeze-fracture images of the subendothelium (Nievelstein et al. 1991) as well as analyses of LDL isolated from atherosclerotic lesions (Hoff and Morton 1985; Guyton and Klemp 1996) indicate that a large proportion of lesional LDL is in the form of aggregate and fused particles averaging approximately 100 nm in diameter. Although the mechanism of LDL aggregation is not known, physiological plausible hypotheses include extensive oxidation (Hoff et al. 1989), hydrolysis by mast cell-derived proteases (Kovanen 1991), and hydrolysis by a Mϕ-derived and endothelial-derived secretory sphingomyelinase (Schissel et al. 1998). Thus, in contrast to the situation with oxLDL, cultured Mϕ incubated with aggregated LDL formed by a variety of methods in vitro accumulate massive amounts of CE.

2.3 Chylomicron Remnants

Post-prandial chylomicron remnants are a class of lipoproteins other than LDL that are epidemiologically associated with atherosclerosis, and are potent inducers of foam cell formation in cultured Mϕ (Havel 1995). Chylomicrons are very buoyant, lipid-rich lipoproteins formed in intestinal epithelial cells during the absorption of dietary fat. Following entry into the circulation, the triglyceride component of the core of chylomicrons is rapidly hydrolyzed by the enzyme lipoprotein lipase bound to the lumenal surface of the endothelium. The resulting chylomicron "remnants" may either be rapidly cleared by the liver or remain in the circulation for an extended period, depending upon specific metabolic and genetic factors that differ among individuals (Havel 1995). Chylomicron remnants remaining in the circulation can become enriched in CE, enter the arterial wall, and enhance lesion development. A widely studied model of CE-rich chylomicron remnants is β-VLDL, a lipoprotein found at very high levels in the plasma of cholesterol-fed rabbits. Chylomicron remnants and β-VLDL lead to marked accumulation of CE when incubated with cultured Mϕ (Goldstein et al. 1980; Mahley et al. 1980).

3 Mechanisms by Which Foam Cells May Accumulate Cholesterol

3.1 Receptor-Mediated Uptake Pathways

Lipoprotein binding to the plasma membrane has been studied with different ligands (e.g., AcLDL, β-VLDL, LDL, oxLDL) in different Mϕ model systems. Generally, Mϕ lipoprotein binding occurs in coated (clathrin-associated) pits on microvillous extensions, and on uncoated regions of plasma membrane ruffles. Both uncoated and coated pits function in the uptake and delivery of bound lipoproteins to lysosomes (van der Schroeff et al. 1983; Traber et al. 1983; Mommaas-Kienhuis et al. 1985). Scavenger receptor-mediated endocytic path-

ways have received the most interest as a mechanism for cholesterol accumulation and Mϕ foam cell formation. Although several proteins may contribute to this overall process, SR-A and CD36 have been demonstrated to play quantitatively significant roles. Recent evidence suggests that SR-A and CD36 represent two scavenging systems that are physiologically important in foam cell formation and atherogenesis.

3.1.1 SR-A

The overexpression or upregulation of SR-A results in the transformation of Chinese hamster ovary cells or peritoneal murine Mϕ into foam cells in the presence of AcLDL (Freeman et al. 1991; de Villiers et al. 1994; de Winther et al. 1999). However, the natural ligand that promotes Mϕ foam cell formation by SR-A in vivo has not been clearly identified, and ligands other than oxidatively modified lipoproteins may lead to foam cell formation by SR-A in vivo (Tabas 1999). SR-A recognizes only extensively modified LDL, and in vitro studies have demonstrated that peritoneal Mϕ lacking SR-A showed 80% less uptake of AcLDL, whereas the uptake of oxLDL was reduced by only 30% (Lougheed et al. 1997; Terpstra et al. 1997). The pro-atherogenic role of SR-A was confirmed in studies with mice deficient in apoE and SR-A (Suzuki et al. 1997). These animals develop 58% less atherosclerotic lesions than apoE-deficient control mice. LDL-receptor/SR-A double-knockout mice similarly showed less atherosclerotic plaque formation (20%) compared with LDL-receptor knockout animals (Sakaguchi et al. 1998).

Mϕ are the primary, but not the only cell type expressing SR-A (Bickel and Freeman 1992; Hughes et al. 1995). Therefore, to dissect the impact of Mϕ SR-A on atherogenesis, mice chimeric for Mϕ SR-A were generated by transplantation with SR-$A^{-/-}$ fetal liver cells. Lethally irradiated C57BL/6 mice reconstituted with SR-$A^{-/-}$ Mϕ and challenged with a high-fat diet for 16 weeks had a 60% reduction in lesion area compared with SR-$A^{+/+}$→C57BL/6 mice (Babaev et al. 2000). $LDLR^{-/-}$ mice reconstituted with SR-$A^{-/-}$ Mϕ and fed a Western diet for 10 weeks had a similar 60% reduction in atherosclerotic lesions compared with SR-$A^{+/+}$→$LDLR^{-/-}$ mice (Babaev et al. 2000). There were no significant differences in serum lipid levels in these two experimental models, suggesting that SR-A does not affect serum cholesterol and triglyceride levels, but plays a central role in atherogenesis by loading vascular wall Mϕ with oxidized and otherwise modified lipoprotein material.

3.1.2
Class B Scavenger Receptors

CD36

CD36, a member of the class B scavenger receptor family, is structurally unrelated to SR-A and more widely expressed, and has a broader ligand specificity (Febbraio et al. 1999). CD36 was originally identified as an oxLDL receptor by an expression cloning strategy used to isolate murine Mϕ receptors that recognize oxLDL, but not AcLDL (Endemann et al. 1993). A genetic polymorphism in the CD36 gene has been identified in Japanese subjects (Kashiwagi et al. 1995) and shown to result in deficient expression of CD36 (NAK^{a-} phenotype). Monocyte-derived Mϕ isolated from these patients bind ≈40% less oxLDL and accumulate ≈40% less cholesterol ester from oxLDL than cells derived from normal controls (Nozaki et al. 1995), further implicating CD36 as a physiological oxLDL receptor. In addition, peritoneal Mϕ from CD36 knockout mice showed a 60% reduction in the uptake of oxLDL and a 52% reduction in the uptake of AcLDL (Febbraio et al. 1999, 2000). ApoE-deficient mice lacking CD36 develop significantly less atherosclerosis with a 77% decrease in aortic tree lesion area (Western diet) and a 45% decrease in aortic sinus lesion area (normal chow) when compared with control $apoE^{-/-}$ mice (Febbraio et al. 2000). These data support an important role for CD36 in foam cell formation and atherogenesis in vivo. However, CD36 also has a crucial role in fatty acid transport in heart and other tissues, and this predominant physiological role of CD36 is unlikely to be attenuated by pharmacological interventions without a marked negative effect on cardiac metabolism.

SR-BI

The other member of the class B scavenger receptor family, SR-BI, also binds oxLDL and LDL, but has been better characterized as a physiologically relevant lipoprotein receptor for HDL (Krieger 2001). SR-BI mediates the selective uptake of HDL CE (Acton et al. 1996; Krieger 1999) by a process in which HDL delivers CE to the cell without lysosomal degradation of the whole HDL particle (Pittman et al. 1987). Recent reports indicate that HDL is internalized by SR-BI and that CE is selectively removed from HDL as HDL particles are recycled back to the cell surface (Silver et al. 2000, 2001). Although CD36 can also mediate the selective uptake of lipids from HDL, it is much less efficient than SR-BI, and its predominant physiological role seems to be in oxLDL, rather than LDL or HDL metabolism (de Villiers et al. 2001). SR-BI is predominantly expressed in liver and steroidogenic tissues, precisely those tissues that show the highest levels of selective uptake of HDL cholesteryl esters (Acton et al. 1996; Krieger 1999). Hepatic overexpression of SR-BI by adenoviral-mediated transfer (Kozarsky et al. 1997; Webb et al. 1998) results in decreased plasma levels of HDL cholesterol and increased biliary cholesterol content.

SR-BI expression can be regulated by estrogen, PPAR agonists, vitamin E, polyunsaturated fatty acids, and cholesterol (Fluiter et al. 1998; Krieger 1999; Spady et al. 1999; Trigatti et al. 2000; Witt et al. 2000). The antiatherogenic role of SR-BI in mouse models of atherosclerosis is unequivocal. The absence of SR-BI in knockout mice dramatically accelerates the onset of atherosclerosis (Trigatti et al. 1999; Huszar et al. 2000), whereas atherosclerosis is suppressed by hepatic overexpression of SR-BI (Arai et al. 1999; Kozarsky et al. 2000; Ueda et al. 2000). Because of the antiatherogenic effects of increased hepatic SR-BI expression, SR-BI represents a possible target for therapeutic intervention in CAD. However, the contribution of Mϕ SR-BI to atherogenesis is unclear and may be a confounding factor.

Selective lipid uptake of CE from HDL has been shown for many cell types, including Mϕ (Stein et al. 1987; Panzenboeck et al. 1997; Hirano et al. 1999; Chinetti et al. 2000). SR-BI expression levels correlate with selective CE uptake from HDL in differentiated THP-1 Mϕ, and caveolin-1, an important constituent of caveolae, acts as a negative regulator (Matveev et al. 1999, 2001). In addition to uptake from HDL, Mϕ also take up CE selectively from LDL (Rinninger et al. 1995) and oxLDL (manuscript submitted). In the latter instance, THP-1 cells selectively internalize CE from oxLDL by a process that appears to be independent of SR-BI. Selective uptake of CE in mouse peritoneal Mϕ has also been suggested in the case of aggregated LDL (Rinninger et al. 1995; Rhainds and Brissette 1999), but the receptors involved have not been identified. It remains to be shown, however, that selective uptake from any of the ligands mentioned can induce Mϕ cholesterol accumulation.

3.1.3 Macrosialin

Murine macrosialin and its human homologue CD68 are extensively glycosylated transmembrane proteins expressed in Mϕ and Mϕ-related cells, including liver Kupffer cells (Rabinowitz and Gordon 1991). Macrosialin is predominantly a late endosomal protein but is also found on the cell surface (Rabinowitz and Gordon 1991; Kurushima et al. 2000). Interest in macrosialin as a scavenger and oxLDL receptor arose when, on the basis of ligand blotting, macrosialin was suggested to bind oxLDL (Ramprasad et al. 1995; Ramprasad et al. 1996). Further examples of evidence supporting a role for macrosialin in modified LDL catabolism include its identification in liver Kupffer cells as the major oxLDL binding protein (van Velzen et al. 1997) and its prominent expression in Mϕ in atherosclerotic plaques from apoE knockout mice (de Villiers et al. 1998). However, we found no binding of oxLDL to macrosialin in intact transfected COS-7 and CHO cells, despite significant cell-surface expression of macrosialin (manuscript submitted). Although macrosialin expression in Mϕ and Kupffer cells is responsive to a pro-atherogenic inflammatory diet and to oxLDL, possibly indicating a compensatory protective role, there is no evidence that macrosialin

functions as an oxLDL receptor on the cell surface or participates in foam cell formation.

3.1.4 LOX-1 and SR-PSOX

More recently, two additional oxLDL receptors were described that are also expressed on Mϕ. Their precise role in foam cell formation and atherogenesis is unclear and is currently the focus of intense investigation. Lectin-like oxLDL receptor-1 (LOX-1) was identified by expression cloning from a cDNA library of cultured bovine aortic endothelial cells (BAECs) and is a type II membrane protein that belongs to the C-type lectin family of molecules (Sawamura et al. 1997). LOX-1 acts as a cell-surface endocytic receptor by mediating the binding, internalization, and proteolytic degradation of oxLDL, but not AcLDL (Moriwaki et al. 1998). Cell-surface LOX-1 can be cleaved through some protease activities that are associated with the plasma membrane, and released into the culture media (Murase et al. 2000). LOX-1 is present in cultured human and murine Mϕ and activated smooth muscle cells (Yoshida et al. 1998); and its expression is inducible by proinflammatory stimuli, such as tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, lipopolysaccharide (LPS), angiotensin II and oxLDL itself (Kume and Kita 2001). In early atherosclerotic lesions in human carotid arteries, LOX-1 is highly expressed in endothelial cells. In more advanced lesions with large atheromatous plaques, LOX-1 expression is more prominent in Mϕ and smooth muscle cells suggesting roles for LOX-1 in foam cell formation and vascular cell dysfunction (Kataoka et al. 2000).

The same group of investigators recently identified another novel cell-surface receptor for oxLDL by expression cloning from phorbol myristate acetate (PMA)-stimulated THP-1 cells, designated SR-PSOX (*s*cavenger *r*eceptor for *p*hosphatidyl*s*erine and *ox*idized lipoprotein). SR-PSOX can specifically bind oxLDL with high affinity, followed by internalization and degradation (Shimaoka et al. 2000). Human and murine SR-PSOX are 30-kDa type I membrane glycoproteins which do not share any homology with other oxLDL receptors, and seem to be identical to the membrane-anchored chemokine CXCL16, which may play a dual role in inflammation and homeostasis (Matloubian et al. 2000). Immunohistochemistry showed that SR-PSOX was predominantly expressed by lipid-laden Mϕ in the intima of atherosclerotic plaques (Minami et al. 2001). Taken together, SR-PSOX may be involved in oxLDL uptake and subsequent foam cell transformation in Mϕ in vivo.

3.1.5 Fatty Acid Binding Protein (aP2)

Another lipid-binding protein that is expressed in both adipocytes and Mϕ also has a role in the development and metabolism of foam cells. Genetic deletion of fatty acid binding protein (aP2), a protein known to have a physiologically im-

portant role in regulating lipid metabolism and insulin sensitivity, has a proatherogenic effect in *ApoE* $^{-/-}$ mice. Isolated Mϕ from *aP2* $^{-/-}$ mice secrete lower levels of inflammatory cytokines and accumulate lower levels of intracellular cholesterol esters in response to modified lipoproteins (Layne et al. 2001; Makowski et al. 2001). The introduction of *aP2* $^{-/-}$ Mϕ to *ApoE* $^{-/-}$x*aP2* $^{+/+}$ mice by bone marrow transplantation results in a reduction of atherosclerotic lesions that is comparable to that observed in *ApoE* $^{-/-}$x*aP2* $^{-/-}$ mice, indicating that it is the Mϕ expression of aP2 that is pro-atherogenic.

3.2 Receptor-Independent Uptake Pathways

In contrast to most other cells, Mϕ also have the capacity to take up materials by phagocytosis. Mϕ phagocytose some types of aggregated LDL that then leads to rapid lysosomal degradation of the accumulated aggregated LDL (Hoff et al. 1989; Hoff and Cole 1991). Two lines of evidence support the phagocytosis of chylomicron remnants and aggregated LDL by Mϕ. First, cytochalasin D inhibited the uptake of lipoprotein particles (Khoo et al. 1988; Suits et al. 1989) and second, electron microscopy studies suggested the accumulation of lipoproteins in phagocytic vacuoles (Salisbury et al. 1985).

Other endocytic pathways have been described that may be unique to Mϕ. Large β-VLDL enter peripheral surface-connected wide invaginations, so-called STEMs (*s*urface *t*ubules for *e*ntry into *M*ϕ), prior to undergoing lysosomal degradation (Tabas et al. 1990; Tabas et al. 1991; Myers et al. 1993). Importantly, there are different fates for β-VLDL cholesterol that enters Mϕ through STEMs and LDL cholesterol that enters Mϕ through a coated pit-mediated endocytic pathway. β-VLDL cholesterol delivered through STEMs leads to more efficient cholesterol esterification compared with LDL cholesterol delivered through coated pits.

Patocytosis is a recently described pathway for human monocyte–Mϕ uptake of aggregated lipoproteins, microcrystalline cholesterol, cholesterol-phospholipid liposomes, and other hydrophobic materials. In this pathway, aggregated LDL induces surface invaginations that connect with a labyrinth of interconnected vacuolar compartments within the Mϕ cytoplasm (Kruth et al. 1999a,b; Zhang et al. 2000). The characteristic hallmark of phagocytosis, namely the pinching off of Mϕ plasma membrane to form a phagocytic vacuole, does not occur in patocytosis. During patocytosis the aggregated LDL accumulates within a cytoplasmic labyrinth that remains connected to the Mϕ surface. While some accumulated aggregated LDL subsequently undergoes lysosomal degradation, most aggregated LDL remains in the surface-connected compartments of the labyrinth. The poor degradation of aggregated LDL taken up by patocytosis differs from the rapid degradation of aggregated LDL taken up by phagocytosis. Actin microfilaments function in lipoprotein uptake during both patocytosis and phagocytosis but not during uptake of β-VLDL into STEMs (Tabas et al. 1990). As a

result, cytochalasin D, an agent that interferes with actin polymerization, inhibits lipoprotein uptake mediated by patocytosis and phagocytosis.

Pinocytosis (uptake of fluid in small vesicles) and macropinocytosis (uptake of fluid in large vacuoles) also potentially function in lipoprotein uptake by Mϕ. In fibroblasts, pinocytotic uptake of LDL in bulk fluid is linearly related to LDL concentration, and the LDL taken up undergoes lysosomal degradation (Goldstein and Brown 1977). A similar pathway appears to function in Mϕ but has not been extensively studied (Traber et al. 1983). Uptake of lipoproteins bound to plasma membrane areas that then form macropinosomes is another mechanism by which lipoproteins may enter Mϕ (Jones et al. 1999; Jones and Willingham 1999). Indeed, AcLDL and aggregated LDL, both potent inducers of cholesterol esterification, engage in prolonged association with surface invaginations in Mϕ (Zha et al. 1997). The underlying mechanism is unknown, but may be quite relevant in foam cell formation in the subendothelium of developing lesions in vivo, where most of the lipoproteins are not free in solution, bur rather are very tightly bound to extracellular matrix, often in an aggregated form.

4
Cholesterol Trafficking Within Macrophages

Multiple pathways exist for lipoprotein and non-lipoprotein sources of cholesterol to enter Mϕ. Following endocytosis of lipoproteins by Mϕ and foam cells, CE is hydrolyzed in lysosomes by a CE hydrolase (Anderson and Sando 1991). The newly liberated FC may either be retained or released from Mϕ, perhaps depending on the pathway by which it was delivered. In most peripheral cells, intracellular levels of FC are controlled in part by an enzymatically regulated cycle of esterification and hydrolysis (Brown et al. 1980). Excess plasma membrane cholesterol enters the cytoplasm and is delivered to the intracellular cholesterol esterifying enzyme, acyl-CoA:cholesterol acyltransferase (ACAT) located predominantly in the endoplasmic reticulum. Esterified cholesterol is stored in membrane-bound inclusions in the cytoplasm. These CE stores can be rehydrolyzed to FC by cytoplasmic neutral cholesterol-ester hydrolases (Khoo et al. 1993) to complete the cycle. Following hydrolysis, FC traffics back to the plasma membrane where it may undergo efflux from the cell.

Two ACAT isoforms have been described: ACAT-1 is expressed largely in peripheral tissues, including Mϕ; and ACAT-2 is expressed in the intestine and liver (Brewer 2000). ACAT inhibitors have been shown to reduce diet-induced atherosclerosis in rabbits and hamsters with minimal alterations to circulating cholesterol levels (Bocan et al. 2001). The mechanism for this effect is presumed to be a reduction in the differentiation of Mϕ into lipid-laden foam cells, which results from the interruption of the cholesterol esterification-hydrolysis cycle (Rodriguez and Usher 2002). An ACAT inhibitor, avasimibe (Pfizer), is now in phase III clinical trials for the treatment of CAD (Insull et al. 2001).

This process of cholesterol trafficking through cells, including Mϕ, is under intense investigation to learn whether it occurs in association with carrier proteins, such as the NPC1 protein (Carstea et al. 1997; Loftus et al. 1997), and whether cholesterol is transported by membrane vesicles or smaller macromolecular complexes which may contain caveolin-1 and other proteins (Smart et al. 1996; Uittenbogaard et al. 2002). Cholesterol and other molecules that become deposited in ceroid inclusions in Mϕ may not be available for eventual cholesterol efflux. These inclusions contain insoluble oxidized and polymerized proteins and lipid presumably including CE. Ceroid inclusions can be produced in vitro when Mϕ take up oxLDL or lipid particles containing lipids that are especially susceptible to oxidation (Ball et al. 1986, 1987, 1988). Sphingomyelin is a cholesterol-binding lipid that exists with cholesterol in endosomes, lysosomes, and in cholesterol-enriched detergent-insoluble membrane microdomains (DIGS) such as caveolae. Sphingomyelin is rapidly hydrolyzed by lysosomal sphingomyelinase, a product of the acid sphingomyelinase gene. Mϕ deficient in acid sphingomyelinase exhibit defective cholesterol trafficking and efflux, suggesting sphingomyelin plays an important role in cholesterol trafficking from intracellular sites to the plasma membrane (Leventhal et al. 2001).

5
Cholesterol Efflux From Macrophages

Mechanisms mediating cholesterol efflux are of critical importance in foam cell development. As with the control of the cholesterol esterification–hydrolysis cycle, many of the mechanisms that are important for the regulation of cholesterol metabolism and efflux in Mϕ and foam cells are shared by the liver and other peripheral tissues. Mϕ have two potential mechanisms for disposing of excess cholesterol: enzymatic modification to more soluble forms, and efflux via membrane transporters.

5.1
Macrophage Excretion of 27-Oxygenated Cholesterol Metabolites

The enzyme cholesterol 27-hydroxylase is expressed in Mϕ at relatively high levels and could potentially play a role in cholesterol excretion by converting it to the more polar and soluble 27-OH-cholesterol and 3β-OH-cholesterolenoic acid (Babiker et al. 1997). In the absence of cholesterol acceptors such as HDL, these compounds are then excreted from Mϕ (Westman et al. 1998; Brown et al. 2000).

5.2
Plasma-Derived HDL

The major mechanism for cholesterol efflux is likely to be via membrane transporters, with HDL serving as a primary extracellular acceptor. This role of HDL is central to the "reverse cholesterol transport" process and may explain the in-

verse correlation of HDL levels with the risk of atherosclerosis (Tall et al. 2000). HDLs induce cholesterol efflux when incubated with Mϕ by stimulating translocation of cholesterol from intracellular membranes to the plasma membrane (Aviram et al. 1989; Bierman et al. 1991). The HDL then acquires excess plasma membrane cholesterol. Alternatively, some studies show that HDL enters Mϕ and acquires cholesterol through interactions with lipid droplets. This cholesterol-enriched HDL is then re-secreted by the Mϕ (Schmitz et al. 1985; Takahashi et al. 1989; Takahashi and Smith 1999).

SR-BI, in addition to mediating selective CE uptake from HDL, has also been shown to promote bi-directional flux of FC and phospholipids between cells and HDL (Ji et al. 1997; Jian et al. 1998; Krieger 1999; Rothblat et al. 1999). The physiologic importance of SR-BI for reverse cholesterol transport is suggested by studies that show mice over-expressing hepatic SR-BI have reduced atherosclerosis (Kozarsky et al. 2000). However, the significance of SR-BI-dependent cellular cholesterol efflux from Mϕ has not been established.

5.3 The Cassette Protein ABCA1

A key insight into the molecular mechanisms responsible for cholesterol efflux resulted from studies of patients with Tangier disease, which is characterized by extremely low levels of HDL and cholesterol accumulation in Mϕ. The cause of Tangier disease was found when several different approaches led to the identification of null mutations in the *ABCA1* (ATP-binding cassette, subfamily A, member 1) gene, encoding a member of the ATP binding cassette family of transporters (Bodzioch et al. 1999; Brooks-Wilson et al. 1999; Lawn et al. 1999; Rust et al. 1999). In vitro studies indicate that ABCA1 mediates transport of cholesterol and phospholipids from cells to apoA-I and other apolipoproteins or to lipid-poor pre-β HDL (Oram et al. 2000; Oram and Lawn 2001). In the absence of sufficient lipidation, nascent HDL particles are rapidly cleared, suggesting a probable explanation for the extremely low HDL cholesterol levels in Tangier patients.

Cholesterol efflux occurs with lipid-free amphipathic apolipoproteins of HDL such as apoA-I that associate with Mϕ phospholipid and form nascent HDL particles. Deletion of apoA-I, the major protein component of HDL, is not sufficient to cause atherosclerosis in mice fed a normal chow diet. However, apoA-I deficiency markedly exacerbates atherosclerosis in hypercholesterolemic mouse models (Voyiaziakis et al. 1998). Adenovirus-mediated overexpression of apoA-I protects against the development of atherosclerosis (Benoit et al. 1999; Tangirala et al. 1999).

In addition to ABCA1, Mϕ and adipocytes also express and secrete the amphipathic apolipoprotein, apoE, in response to increases in intracellular cholesterol. Importantly, apoE expression in arterial wall Mϕ is believed to promote cholesterol efflux from lipid-laden foam cells, and to protect against atherogenesis by a mechanism that is distinct from its well-known role in hepatic lipopro-

tein uptake. Mϕ produce their own HDL particles that may mediate Mϕ cholesterol efflux through an autocrine/paracrine mechanism involving both apoE and ABCA1. This occurs when Mϕ secrete apoE that associates with Mϕ phospholipid to form apoE-phospholipid discoidal complexes (Basu et al. 1982). These apoE–phospholipid particles acquire cholesterol from Mϕ sufficiently to cause a decrease in cholesterol content of human monocyte-derived Mϕ and cyclic adenosine monophosphate (cAMP)-treated RAW mouse Mϕ, but not untreated mouse peritoneal Mϕ (Smith et al. 1996; Zhang et al. 1996). ABCA1 probably plays a major role in this cellular efflux of cholesterol and phospholipid to apoE. Mϕ-specific expression of human apoE reduces atherosclerosis in hypercholesterolemic apoE-null mice, supporting a possible function of Mϕ-produced apoE within lesions in promoting cholesterol efflux (Bellosta et al. 1995).

Recent studies link ABCA1, PPARs, apoE and the nuclear receptors LXRα and LXRβ in cholesterol efflux. These nuclear receptors are important in regulating the expression of various proteins involved in the control of hepatic lipid metabolism, and have significant biological effects on the regulation of systemic lipid and cholesterol levels, as well as on the regulation of cellular cholesterol efflux. Among the main physiological activators of the LXR receptors are oxysterols, and genetic deletion of LXRα in mice attenuates the ability of the liver to regulate the expression of lipid-metabolizing gene products in response to dietary cholesterol (Peet et al. 1998). In contrast, mice in which LXRβ is deleted respond normally to changes in dietary cholesterol (Alberti et al. 2001). These receptors might also have an important role in cholesterol efflux, independent of their role in lipid metabolism. Overexpression of LXRα in fibroblast or Mϕ cell lines, or treatment of these cells with oxysterols, induces expression of the mRNA for ABCA1, indicating that its expression might be under the control of oxysterols through their interaction with LXR (Venkateswaran et al. 2000). Cholesterol regulation of Mϕ and adipocyte expression of apoE is also under the transcriptional control of LXR receptors. It has recently been shown that adipocytes and Mϕ from *Lxrα* $^{-/-}$, *Lxrβ* $^{-/-}$ mice and double-knockout mice have reduced or absent activation of apoE expression by oxysterols. These data indicate that Mϕ LXR receptors are physiologically important in the regulation of cholesterol efflux (Laffitte et al. 2001).

The PPAR class of transcription factors belongs to the broad nuclear hormone receptor superfamily, which includes the steroid, retinoid, and thyroid hormone receptors. The three PPAR isoforms (α, δ, and γ) form a heterodimer with RXR and regulate the transcription of genes that are involved in lipid and glucose metabolism. Binding of lipid ligands to the PPAR–RXR heterodimer activates the transcription factor complex (Kersten et al. 2000). PPAR-γ is most abundantly expressed in adipocytes, but is also present in Mϕ (Ricote et al. 1998). PPAR-γ is the specific target of the insulin-sensitizing thiazolidinediones that are widely used in the treatment of hyperlipidemia and type 2 diabetes mellitus. A role for PPAR-γ in foam cell formation became relevant when it was shown that lipid ligands present in oxLDL, as well as the thiazolidinediones,

could activate PPAR-γ in Mϕ, upregulate expression of the oxLDL receptor, CD36, and increase their own uptake (Nagy et al. 1998; Tontonoz et al. 1998). These findings, describing a forward feeding loop resulting in increased oxLDL uptake and foam cell formation, raised serious concerns about the potential long-term side effects of thiazolidinedione treatment of type 2 diabetic patients, who are already at increased risk for developing atherosclerosis (Spiegelman 1998). However, recent studies show a more complex regulation of Mϕ lipid metabolism by PPAR-γ resulting in protection from atherosclerosis. In addition to upregulating CD36 expression, troglitazone treatment of peritoneal Mϕ also downregulates SR-A expression (Moore et al. 2001). This opposing regulation of modified LDL receptors results in the largely unchanged uptake of oxLDL by Mϕ. Studies in apoE and LDL-receptor knockout mice showed that treatment with PPAR-γ agonists (rosiglitazone and troglitazone) had a favorable influence on the development of atherosclerosis (Li et al. 2000; Chen et al. 2001; Collins et al. 2001). PPAR-γ was shown to limit cholesterol accumulation in Mϕ by increasing ABCA1 expression and cholesterol efflux in response to apoA-I. Interestingly, the PPAR-γ induction of ABCA1 appears to be driven through the PPAR-γ induction of the nuclear receptor LXR (Chawla et al. 2001; Chinetti et al. 2001). A major physiologic role of PPAR-γ/LXR signaling may, therefore, be modulation of the reverse cholesterol transport process in Mϕ and the atheroprotective actions of PPAR-γ agonists may be partly related to the induction of cholesterol efflux. Recent data also indicate that selective activation of another PPAR isoform, PPAR-δ, resulted in lipid accumulation in primary Mϕ and THP-1 cells (Vosper et al. 2001). A selective PPAR-δ agonist increased SR-A and CD36 expression, and downregulated gene expression of cholesterol 27-hydroxylase and apoE. The exact significance of PPAR-δ activation as a promoter of Mϕ foam cell formation remains unclear.

6 Foam Cell Death

Foam cell death may interfere with removal of cholesterol from atherosclerotic lesions and contribute to the accumulation of extracellular cholesterol in lesions. There are conflicting reports whether massive cholesterol accumulation within Mϕ actually causes foam cell death. Cholesterol accumulation in Mϕ treated with an ACAT inhibitor is associated with the buildup of unesterified cholesterol in cellular membranes and Mϕ cell death (Warner et al. 1995; Kellner-Weibel et al. 1998). However, Mϕ accumulation of excessive unesterified cholesterol does not always result in foam cell death. Mϕ are able to increase phospholipid content, a natural buffer, in response to excess unesterified cholesterol (Tabas 1997). Even when maintaining normal phospholipid content, human monocyte-derived Mϕ are able to accumulate large amounts of cholesterol crystals without displaying cell death. This may be because these Mϕ sequester excessive cholesterol in protective locations such as surface-connected compartments or lysosomes. The conversion of cholesterol to oxysterols could be anoth-

er factor determining the toxicity of excess cholesterol, because oxysterols, but not cholesterol, are reported to be toxic to human monocyte-derived Mϕ (Clare et al. 1995).

7 Therapeutic Implications

The data describing the importance of Mϕ expression of various types of scavenger receptors, ACAT, PPARs, LXR, ABCA1, apoE and aP2 for the progression of atherosclerosis in mouse models confirm the central contribution of lipid metabolism in Mϕ and foam cells to the pathology of CAD. Therapeutic interventions aimed at inhibiting the pathways that are involved in the accumulation of lipid by Mϕ as they differentiate into foam cells could complement both existing and developing therapies for the management of lipid and lipoprotein levels.

Important caveats for the benefit of these approaches may be the impact of a particular therapeutic intervention on the normal function of the immune system and its response to invading pathogens, as is evident from the described multi-functional role of scavenger receptors, particularly SR-A in innate immunity (Platt and Gordon 2001). As existing lipid-lowering therapies have an excellent risk/benefit ratio, new therapies for the treatment of CAD will require a similarly excellent safety profile in order to be maximally accepted and used. Significant impairment of the normal immune system, leading to an increase in the rate of infections, will probably be unacceptable for therapies used for the treatment of CAD.

8 Conclusion

The Mϕ foam cell is a prominent and important component of the atherosclerotic lesion, playing roles in both lesion initiation and lesion progression. Foam cell biology as it pertains to both of these processes can be understood only through analysis of how Mϕ interact with and internalize atherogenic lipoproteins and how they metabolize lipoprotein-derived cholesterol. Much of our knowledge of foam cell formation comes from studying cultured Mϕ, often permanent cell lines, interacting with monomeric lipoproteins dissolved in tissue culture medium. Mϕ subtypes are known to differ in important ways, and lesional Mϕ may in fact possess important differences to those studied in the laboratory. Likewise, the form of lipoprotein that interact with Mϕ in lesions is almost certainly different from those investigated in most cell-culture studies. Therefore, studies examining the interaction of Mϕ with aggregated lipoproteins should be pursued in future research.

The conclusion that foam cells promote atherogenesis may require careful examination. Clearly, Mϕ foam cells can secrete molecules, such as oxidants, growth factors, inflammatory cytokines, and metalloproteinases, which may promote lesion development and plaque breakdown. But the ability of Mϕ to

scavenge potentially harmful molecules, including oxidized lipids, may be beneficial, such as is often the case in other types of inflammatory and infectious lesions. Thus, as we identify specific molecules related to foam cell biology, and as we increasingly use in vivo systems, such as transgenic and knockout mice, to study these molecules, experimental strategies must specifically address this critical issue. Only through such studies will we be able to use our knowledge of foam cell biology to rationally design anti-atherogenic therapeutic interventions.

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Dietary Fatty Acids and Macrophages

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Abstract Fatty acids constitute key components of cell membranes. The fatty acid composition of cell membranes influences membrane fluidity. Membrane phospholipids are substrates for the generation of intracellular and extracellular signalling molecules. The supply of fatty acids to monocytes and macrophages influences their fatty acid composition. Thus, dietary fatty acid composition influences that of monocyte and macrophage membranes. This can have functional consequences. The *n*-6 polyunsaturated fatty acid (PUFA) arachidonic acid is the principal substrate for generation of eicosanoids via cyclooxygenase and lipoxygenase enzymes. Increased availability of *n*-3 PUFAs (found in oily fish and fish oil) can affect chemotaxis, phagocytosis, respiratory burst, eicosanoid production, cytokine production and other monocyte/macrophage functions. Although some of the effects of *n*-3 PUFAs may be brought about by modulation of the amount and types of eicosanoids made, it appears that these fatty acids might elicit some of their effects by eicosanoid-independent mechanisms, including actions upon intracellular signalling pathways and transcription factor activity. The functional effects of *n*-3 PUFAs are generally termed as "anti-inflammatory" and are considered beneficial to health. The effects of dietary fatty acids on monocyte/macrophage function may also be relevant to atherosclerosis, which is now recognised to include an inflammatory component. Fatty acids could potentially affect the degree of oxidation of low-density lipoprotein (LDL), its uptake by vascular cells, aspects of foam cell formation and inflammatory activity within atherosclerotic lesions. These effects might account for the reported protective effects of *n*-3 PUFAs towards cardiovascular mortality.

Keywords Cardiovascular disease, Cholesterol, Cytokine, Eicosanoid, Fish oil, Foam cell, LDL oxidation, Phagocytosis, Polyunsaturated fatty acid, Respiratory burst

Abbreviations

AA	Arachidonic acid
COX	Cyclooxygenase
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
GLA	γ-Linolenic acid
IFN	Interferon
IL	Interleukin
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
MUFA	Monounsaturated fatty acid
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
TNF	Tumour necrosis factor

1
Dietary Fatty Acids

1.1
Types and Sources of Fatty Acids in the Human Diet

In Western countries an adult eats on average 75–150 g of fat each day and fat contributes 30%–45% of dietary energy. By far the most important component of dietary fat in quantitative terms is triacylglycerol, which in most diets constitutes more than 95% of dietary fat. Each triacylglycerol molecule is composed of three fatty acids esterified to a glycerol backbone. Thus, fatty acids are major constituents of dietary fat. Because of the wide range of foods consumed, the human diet contains a great variety of fatty acids. It is the nature of the constituent fatty acids (their chain length and degree of unsaturation) that gives a fat its physical properties.

Fatty acids have systematic names, but most also have common names and are described by a shorthand nomenclature, e.g. 18:2*n*-6. This nomenclature indicates the number of carbon atoms in the hydrocarbon chain, the number of double bonds in the hydrocarbon chain, and the position of the first double bond from the methyl terminus of the chain (see Fig. 1). It is the *n*-7, *n*-9, *n*-6 or *n*-3 notation that indicates the position of the first double bond in the hydrocarbon chain for a fatty acid. Thus, an *n*-6 fatty acid has the first double bond on carbon number 6 from the methyl terminus and an *n*-3 fatty acid has the first double bond on carbon number 3 from the methyl terminus. The *n*- notation is sometimes referred to as ω or omega.

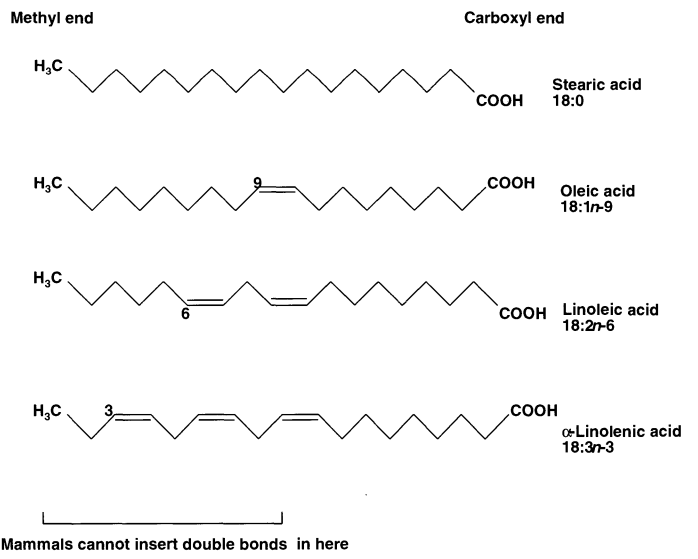

Fig. 1 The structure of fatty acids

Saturated fatty acids and most monounsaturated fatty acids (MUFAs) can be made in mammalian tissues from non-fat precursors, but this does not usually occur in humans eating a Western diet, since the consumption of fat in general, and of saturated and monounsaturated fatty acids in particular, is high. However, mammals cannot insert double bonds between the methyl terminus and carbon number 9 in oleic acid (18:1*n*-9) (Fig. 1). Thus, mammals cannot convert oleic acid into linoleic acid (18:2*n*-6). The Δ12-desaturase enzyme which does this is found only in plants. Likewise, mammals cannot convert linoleic acid into α-linolenic acid (18:3*n*-3). The Δ15-desaturase enzyme which does this is again found only in plants. Since these two fatty acids cannot be made by mammals they are termed essential fatty acids. Also, because mammalian tissues do not contain the Δ15-desaturase, they cannot interconvert *n*-6 and *n*-3 fatty acids. Plant tissues and plant oils tend to be rich sources of linoleic and α-linolenic acids. These fatty acids are the main polyunsaturated fatty acids (PUFAs) in most human diets: the average intake of linoleic acid among adult males in the United Kingdom is approximately 14 g/day, while that of α-linolenic acid is approximately 2 g/day. These intakes are lower than those of saturated and monounsaturated fatty acids (approximately 42 and 32 g/day, respectively).

1.2 Synthesis of Longer Chain Polyunsaturated Fatty Acids

Once consumed in the diet, linoleic acid can be converted via γ-linolenic acid (GLA; 18:3*n*-6) and dihomo-γ-linolenic acid (DGLA; 20:3*n*-6) to arachidonic acid (AA; 20:4*n*-6) by the pathway outlined in Fig. 2. Using the same pathway (Fig. 2), dietary α-linolenic acid can be converted into eicosapentaenoic acid (EPA; 20:5*n*-3), docosapentaenoic acid (22:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3). Thus, there is competition between the *n*-6 and *n*-3 fatty acids for the enzymes which metabolise them. The long chain n-3 PUFAs, EPA and DHA can be obtained directly from the diet since they are found in relatively high proportions in the tissues of so-called "oily fish" (e.g. herring, mackerel, tuna, sardines) and in the commercial products called "fish oils" which are a preparation of the body oils of oily fish; EPA and DHA are also found in high proportions in the oils extracted from the livers of some other species of fish (e.g. cod). EPA and DHA comprise 20%–30% of the fatty acids in a typical preparation of fish oil. The intake of longer chain *n*-3 PUFAs is not clearly known, but it appears that the average adult in the United Kingdom consumes about 250 mg EPA plus DHA per day. In the absence of significant consumption of oily fish, α-linolenic acid is the major dietary *n*-3 fatty acid.

1.3 Polyunsaturated Fatty Acid Synthesis by Macrophages

Experiments with murine peritoneal macrophages in culture demonstrated that these cells have limited capacity to carry out the key metabolic transformations

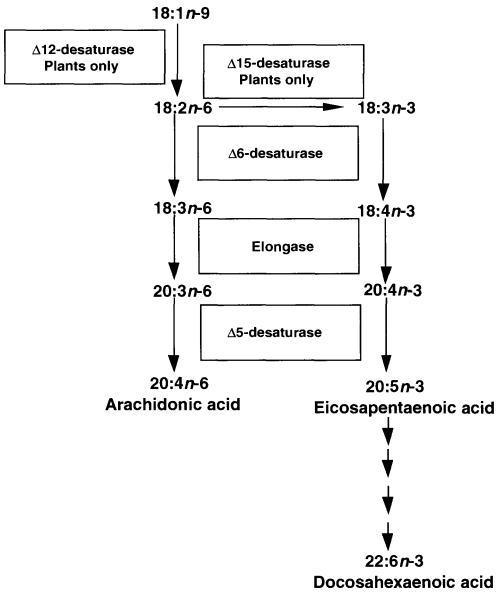

Fig. 2 Outline of the pathway of biosynthesis and metabolism of polyunsaturated fatty acids

shown in Fig. 2. These studies indicate that (murine) macrophages have an efficient fatty acid elongase (capable of converting linoleic acid to 20:2n-6, GLA to DGLA and EPA to 22:5*n*-3), lack Δ6-desaturase activity and have a low activity of Δ5-desaturase (Chapkin et al. 1988a,b; Chapkin and Coble 1991). Thus, the bulk, if not all, of the AA, EPA and DHA in macrophage phospholipids is likely to originate from exogenous sources. PUFAs circulate in the bloodstream as the fatty acid components of phospholipids and triacylglycerols in lipoproteins and as non-esterified fatty acids. These circulating lipid pools provide PUFAs to immune cells such as monocytes and macrophages.

2
Roles of Fatty Acids in Monocytes and Macrophages

The majority of the fatty acids taken up by cultured macrophages are incorporated into phospholipids of the plasma and organelle membranes. There is selective enrichment of particular phospholipid classes with particular fatty acids (e.g. AA is particularly associated with phosphatidylcholine). The fatty acid components of membrane phospholipids are partly responsible for regulating the fluidity of the membrane (Stubbs and Smith 1984). Fluidity ensures the appropriate environment for the function and movement of membrane proteins, and changing fluidity can affect the activities of such proteins (Stubbs and Smith 1984). Membrane phospholipids are also the source of a range of signalling molecules including inositol-1,3,5-trisphosphate, diacylglycerol, phosphatidic acid, lysophosphatidylcholine, choline, ceramide, platelet activating factor and AA. It is now recognised that there are particular regions of the cell

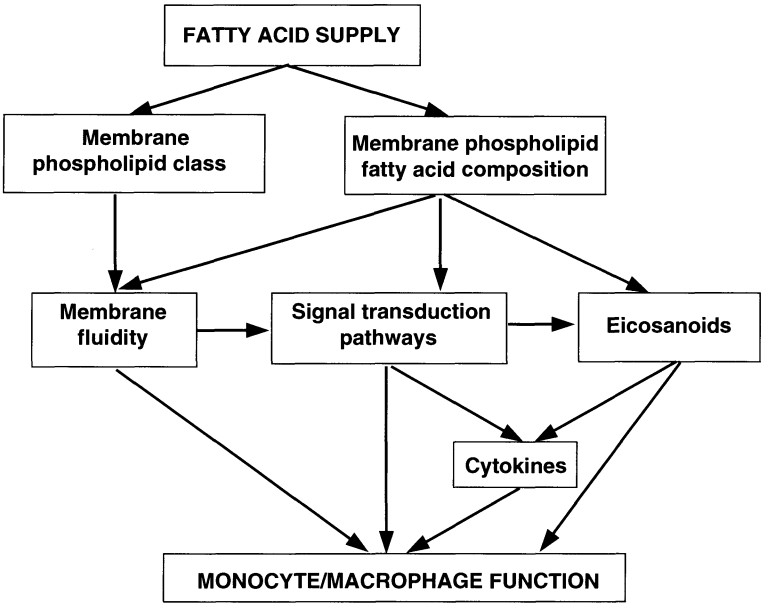

Fig. 3 Mechanisms whereby polyunsaturated fatty acids might exert effects on monocyte/macrophage function

membrane specialised for these roles in cell signalling; these regions are termed rafts, and they are sites where certain receptors and signalling enzymes are clustered (Simons and Toomre 2000). Rafts are also characterised by the presence of a different range of phospholipids from the bulk of the plasma membrane, and this most likely relates to providing the correct environment for the receptors and signalling enzymes and providing substrates for the generation of signalling molecules. The arrangement of the phospholipids in membranes and their fatty acid compositions might have important functional effects for macrophages (Fig. 3).

3 Influence of Altered Supply of Fatty Acids on Monocyte and Macrophage Fatty Acid Composition

Monocyte and macrophage membrane phospholipids are rich in AA, which typically comprises 15%–25% of the fatty acids. Since it appears that these cells have a limited capacity to synthesise AA from linoleic acid (see "Polyunsaturated Fatty Acid Synthesis by Macrophages" above), the AA most likely originates from metabolism of linoleic acid in the liver. The proportions of EPA and DHA in macrophages and monocytes are low, since most diets contain little α-linolenic acid and very little EPA and DHA.

Culture of macrophages with a given fatty acid results in enrichment of that fatty acid in the cells (Calder et al. 1990). The incorporation of exogenously supplied *n*-3 PUFAs (EPA and DHA) is frequently at the partial expense of AA, i.e. increased proportions of *n*-3 PUFAs result in a decreased proportion of AA. This is believed to be of functional significance because of the role of AA as an eicosanoid precursor (see "Arachidonic Acid as an Eicosanoid Precursor" below). Furthermore, changes in macrophage membrane fluidity occur as a result of selective enrichment with certain fatty acids (Mahoney et al. 1980), and this might affect functions such as phagocytosis (see "Phagocytosis" below).

Changing the fatty acid composition of the diet has also been demonstrated to result in a change in the fatty acid composition of macrophages. Feeding mice a diet in which borage oil (contains GLA) was the fat source resulted in a marked increase in the proportion of DGLA in peritoneal macrophage phospholipids (Chapkin et al. 1988c). Feeding rats a diet in which linseed oil was the fat source resulted in a marked increase in the proportions of α-linolenic acid, EPA, docosapentaenoic acid and DHA in peritoneal macrophage phospholipids (Magrum and Johnston 1983). Feeding laboratory animals a diet rich in fish oil results in a marked increase in the proportions of EPA and DHA in macrophage phospholipids (see Calder 1998 for references). The incorporation of *n*-3 PUFAs following feeding of either linseed or fish oil results in a decline (up to 50%) in the proportion of AA in macrophage phospholipids.

Those studies that have examined the effects of providing fish oil to humans on the fatty acid composition of peripheral blood monocytes show a marked increase in the proportion of EPA (e.g. from <0.1 to >1.5% [Lee et al. 1985; Fisher et al.1990]) and DHA. The incorporation is paralleled by a decline in the proportion of AA (e.g. from 22.6% to 15% [Fisher et al. 1990]). The incorporation of dietary EPA and DHA from the diet into human monocytes reaches a plateau within 3 or 4 weeks (see Gibney and Hunter 1993; Yaqoob et al. 2000). However, both the time course of incorporation and the extent of the compositional change depend upon the dose of fish oil provided.

4
Regulation of Monocyte and Macrophage Functions by Dietary Fatty Acids

4.1
Influence of Fatty Acids on Membrane-Mediated Functions of Monocytes and Macrophages

4.1.1
Chemotaxis

Chemotaxis of monocytes and macrophages could be affected by changes in the fatty acid composition of membrane phospholipids which might influence the binding of chemotactic agents to their receptors, the subsequent signalling pathways, or the cytoskeletal rearrangements which occur. Chemotaxis of blood

monocytes towards the chemoattractants leukotriene (LT) B_4 and formyl-methionyl-leucyl-phenylalanine was found to be suppressed following supplementation of the human diet with approximately 5.5 g EPA plus DHA per day for 6 weeks (Lee et al. 1985; Endres et al. 1989; Schmidt et al. 1992). There was no effect of a much lower dose of *n*-3 PUFAs (0.65 g/day for 12 weeks) upon monocyte chemotaxis towards pooled human serum (Schmidt et al. 1996).

4.1.2 Phagocytosis

The ability of a cell to perform phagocytosis may be influenced by membrane structure, in particular by the fluidity of the membrane, which may in turn be modulated by the fatty acid composition of membrane phospholipids (Mahoney et al. 1980). Indeed, several studies show that phagocytosis by murine macrophages is influenced by manipulation of their fatty acid composition in culture (see Calder 1998 for references). In general, increasing the macrophage content of saturated fatty acids decreases the ability to perform phagocytosis, while increasing the macrophage content of PUFAs increases the ability to perform phagocytosis. These studies showed phagocytosis to be highly correlated with the degree of phospholipid fatty acid unsaturation, suggesting that membrane fluidity is an important determinant of phagocytosis.

Despite the consistency of the effects of fatty acids on phagocytosis by macrophages following their enrichment with fatty acids in culture, most studies show little or no effect of dietary fatty acid manipulation on phagocytosis by rodent and pig macrophages (see Calder 1998 for references) or human monocytes (Halvorsen et al. 1997; Thies et al. 2001). The differences between the effects of fatty acids delivered in vitro or through the diet most likely relate to the much smaller changes in fatty acid composition observed in the latter case.

4.1.3 Respiratory Burst

There is a large but inconsistent literature on the effects of dietary fish oil on respiratory burst by macrophages. This might in part relate to the different experimental models used, particularly the stimulus used to generate respiratory burst, and to the different ways of expressing the results from such measurements. Some studies demonstrate that feeding fish or linseed oils results in a reduction in the absolute amount of superoxide (and hydrogen peroxide) generated at a given time after stimulation of macrophages with some agents (see Calder 1998 for references). However, Eicher and McVey (1995) reported no effect of dietary fat on the number of murine Kupffer cells engaging in respiratory burst. A detailed study of the hydrogen peroxide generation by peritoneal macrophages from mice fed safflower or fish oil was conducted by Hubbard et al. (1991). These authors found that macrophages from fish oil-fed mice showed lower hydrogen peroxide production in response to unopsonised zymosan, but

production in response to phorbol ester was not different from cells of mice fed the different fatty acids. However, macrophages from fish oil-fed mice produced more hydrogen peroxide than those from safflower oil-fed mice following priming with a high concentration of interferon (IFN)-γ (Hubbard et al. 1991). Thus the true impact of dietary *n*-3 PUFAs on respiratory burst remains unclear. There have been few investigations of the influence of dietary fatty acids on respiratory burst by human monocytes. Fisher et al. (1990) reported that giving healthy volunteers 6 g EPA plus DHA per day for 6 weeks resulted in a marked decrease in the production of superoxide by zymosan-stimulated monocytes. In contrast, Halvorsen et al. (1997) reported no effect of 3.8 g EPA or DHA per day for 7 weeks on superoxide production by monocytes in response to *Escherichia coli*. Furthermore, superoxide production by monocytes in response to *E. coli* was not affected by consumption of 2 g α-linolenic acid, 0.75 g DHA or 1.2 g EPA plus DHA per day for 12 weeks by healthy elderly humans (Thies et al. 2001). These data suggest that there is little impact of modestly increased consumption of *n*-3 PUFAs on respiratory burst by human monocytes.

4.2 Influence of Fatty Acids on Eicosanoid Generation by Monocytes and Macrophages

4.2.1 Arachidonic Acid as an Eicosanoid Precursor

Eicosanoids are a family of oxygenated derivatives of DGLA, AA and EPA. Eicosanoids include prostaglandins (PGs), thromboxanes, LTs, lipoxins, hydroperoxyeicosatetraenoic acids and hydroxyeicosatetraenoic acids. Monocytes and macrophages are important sources of eicosanoids. Because the membranes of monocytes and macrophages typically contain large amounts of AA, compared with DGLA and EPA, AA is usually the principal precursor for eicosanoid synthesis. AA in the monocyte/macrophage can be mobilised by various phospholipase enzymes, most notably phospholipase A_2, and the free AA can subsequently act as a substrate for cyclooxygenase (COX), forming 2-series PGs and related compounds, or for one of the lipoxygenase (LOX) enzymes, forming 4-series LTs and related compounds (Fig. 4). There are two forms of COX: COX-1 is a constitutive enzyme and COX-2 is induced in response to stimulation, for example with bacterial lipopolysaccharide (LPS) or tumour necrosis factor (TNF), and is responsible for the marked elevation in production of PG which accompanies such cellular activation. Monocytes and macrophages produce large amounts of PGE_2 and $PGF_{2\alpha}$. The LOX enzymes have different tissue distributions, with 5-LOX being the most important in immune cells including monocytes and macrophages.

4.2.2
Effects of Eicosanoids on Inflammation and Immunity

Eicosanoids are involved in modulating the intensity and duration of inflammatory and immune responses. The effects of PGE_2 and LTB_4 have been studied most widely. PGE_2 has a number of pro-inflammatory effects including inducing fever, increasing vascular permeability and vasodilation and enhancing pain and oedema caused by other agents such as histamine. PGE_2 acts on T cells to suppress proliferation and interleukin (IL)-2 and IFN-γ production. PGE_2 also inhibits natural killer cell activity. Thus, in these respects PGE_2 is immunosuppressive. With respect to cytokine production by monocytes and macrophages, PGE_2 inhibits production of TNF-α, IL-1 and IL-6. Since TNF-α induces COX-2 and so promotes PGE_2 production, the inhibition of synthesis of the classic pro-inflammatory cytokines by PGE_2 forms an important regulatory loop. LTB_4 increases vascular permeability, enhances local blood flow, is a potent chemotactic agent for leukocytes (including monocytes), induces release of lysosomal enzymes by neutrophils, enhances generation of reactive oxygen species, inhibits lymphocyte proliferation and promotes natural killer cell activity. The 4-series LTs also regulate production of pro-inflammatory cytokines; for example LTB_4 enhances production of TNF-α, IL-1 and IL-6. In this latter respect, PGE_2 and LTB_4 are antagonistic. Thus, AA gives rise to mediators which can have opposing effects to one another, so the overall physiological effect will be governed by the concentration of those mediators, the timing of their production and the sensitivities of target cells to their effects.

4.2.3
EPA as an Alternative Eicosanoid Precursor

Since increased consumption of fish oil results in a decrease in the amount of AA in the membranes of monocytes and macrophages (see "Influence of Altered Supply of Fatty Acids on Monocyte and Macrophage Fatty Acid Composition" above), there will be less substrate available for synthesis of eicosanoids from AA. Furthermore, *n*-3 PUFAs inhibit phospholipase A_2 activity in macrophages, competitively inhibit the oxygenation of AA by COX, and inhibit the cytokine-induced upregulation of COX-2 gene expression (Curtis et al. 2000). Thus, fish oil feeding results in a decreased capacity of monocytes and macrophages to synthesise eicosanoids from AA. This has been demonstrated in a variety of animal models and following high-dose fish oil feeding in humans (see Calder 1998 for references).

In addition to effects on generation of eicosanoids from AA, EPA is able to act as a substrate for both COX and 5-LOX (Fig. 4), giving rise to derivatives which have a different structure from those produced from AA (i.e. 3-series PGs and 5-series LTs). Thus, the EPA-induced suppression in the production of AA-derived eicosanoids can potentially be accompanied by an elevation in the production of EPA-derived eicosanoids. Studies in experimental animals have dem-

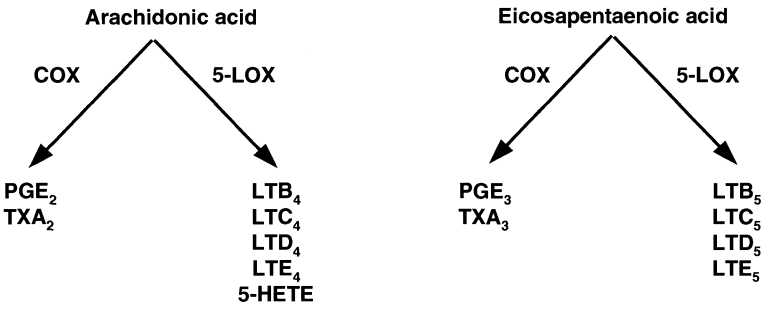

Fig. 4 Generation of eicosanoids from polyunsaturated fatty acids

onstrated that feeding fish oil results in markedly enhanced production of 5-series LT (Chapkin et al. 1990; Whelan et al. 1991). Similarly, dietary fish oil (at a high dose) was demonstrated to significantly increase generation of LTB_5, 6-trans LTB_5 and 5-hydroxyeicosapentaenoic acid by stimulated human monocytes (Lee et al. 1985; Sperling et al. 1993). The generation of EPA-derived COX metabolites following fish oil feeding has not been demonstrated suggesting that, at the concentrations incorporated into membrane phospholipids, EPA is a relatively poor substrate for COX. It is considered that the eicosanoids produced from EPA are less biologically potent than the analogues synthesised from AA, although the full range of biological activities of these compounds has not been investigated.

4.3 Influence of Fatty Acids on Cytokine Generation by Monocytes and Macrophages

Since *n*-3 PUFAs decrease PGE_2 production and since PGE_2 is an inhibitor of inflammatory cytokine production, it would be predicted that *n*-3 PUFAs would enhance production of TNF, IL-1 and IL-6. Some animal studies support this prediction as far as TNF and IL-1 are concerned (see Calder 1998 for references). However, other animal studies demonstrate the reverse effect of fish oil feeding: significantly decreased production of TNF, IL-1 and IL-6 following LPS stimulation of macrophages from fish oil-fed rodents has been reported (see Calder 1998 for references). The studies showing reduced cytokine production after fish oil feeding are supported by cell culture studies which demonstrate that EPA or DHA decrease the production of TNF-α, IL-β and tissue factor by monocytic cell lines (Baldie et al. 1993; Chu et al. 1999), and that *n*-3 PUFAs inhibit the cytokine-induced up-regulation of the TNF-α and IL-1α genes in bovine chondrocytes (Curtis et al. 2000). Whatever the reasons for the differences between study outcomes, it is evident that *n*-3 PUFAs might affect cytokine production by mechanisms other than a decrease in production of PGE_2.

A large number of studies have now investigated the effect of dietary *n*-3 PUFAs on ex vivo cytokine production by LPS-stimulated blood mononuclear cells or purified monocytes from healthy human subjects (reviewed by Calder

2001a). A high dose of α-linolenic acid (approximately 15 g/day for 4 weeks) decreased by about 30% the production of IL-1β and TNF-α production by LPS-stimulated human mononuclear cells (Caughey et al. 1996). A lower dose of α-linolenic acid (2 g/day for 12 weeks) did not affect production of TNF-α, IL-1β or IL-6 by such cells (Thies et al. 2001). A number of studies have shown that supplementation of the diet with between 2.4 and 5 g EPA plus DHA per day for a number of weeks leads to a significant reduction in ex vivo production of TNF, IL-1 and IL-6 (e.g. Endres et al. 1989; Meydani et al. 1991; Caughey et al. 1996; see Calder 2001a,b for further references). Similarly high dose fish oil (providing 4.6 g EPA plus DHA/day) resulted in decreased expression of mRNA for platelet-derived growth factors A and B and monocyte chemoattractant protein-1 in unstimulated and adherence-stimulated human monocytes (Baumann et al. 1999). Lower doses of EPA plus DHA appear to be without effect on cytokine production (Schmidt et al. 1996; Blok et al. 1997; Thies et al. 2001).

5
Dietary Fatty Acids and Atherosclerosis

5.1
Dietary Fatty Acids, Blood Cholesterol and Cardiovascular Disease

Atherosclerosis is the leading cause of death in Western populations. One of the few statements concerning the condition that can be made with certainty is that blood cholesterol concentrations play an important role. Blood cholesterol represents one of the "modifiable" risk factors for cardiovascular disease, which means that it can be altered through pharmacological or dietary therapy. The degree to which treatment is able to alter blood cholesterol levels depends to some extent on an individual's "non-modifiable" risk factors, which include family history, race, age and gender. Epidemiological studies (such as the Multiple Risk Factor Intervention Trial 1982) and drug trials suggest that a lowering of blood cholesterol concentration by 1% decreases the risk of a heart attack by 2% (Vines 1989). In most individuals, low-density lipoprotein (LDL) contributes approximately 75% of total circulating cholesterol. With the elucidation of the LDL and scavenger receptor pathways and the discovery that oxidation of LDL is a pre-requisite for uptake by macrophages, a widely accepted hypothesis of the mechanisms underlying atherosclerosis has evolved, whereby an elevated LDL-cholesterol concentration predisposes towards oxidation and subsequent uptake of modified LDL by macrophages (Kruth 2001).

Although dietary cholesterol is involved in blood cholesterol homeostasis, it generally has little impact on total cholesterol concentration, since homeostasis primarily involves regulation of hepatic synthesis and degradation of cholesterol. Dietary fat, especially saturated fat, intake, on the other hand, is correlated with total blood cholesterol concentration in cross-sectional studies of populations with markedly different total fat intakes (Keys 1970). However, while cross-cultural studies tend to find significant associations between dietary fat

consumption and blood cholesterol concentration, within-population studies often fail to find this correlation, probably because the variation in fat intake is much smaller (Caggiula and Mustad 1997). Nevertheless, the relationships between intake of each class of dietary fatty acid, blood cholesterol and cardiovascular disease continue to be a subject for debate. The general consensus is that the relationship between dietary fatty acids and coronary heart disease is mediated in part by effects on blood cholesterol concentrations, and, while saturated fatty acids tend to be positively associated with both blood cholesterol and coronary heart disease, relationships between other fatty acids (MUFAS and PUFAs) are less consistent, but tend to be negative (Caggiula and Mustad 1997).

Cholesterol-lowering drug intervention trials, such as those testing the effects of statins, prove beyond doubt that decreasing blood cholesterol concentration in individuals with existing coronary heart disease or with a raised cholesterol concentration reduces the risk of a coronary event. However, paradoxically, while blood cholesterol concentration is a primary risk factor, it does not serve as an adequate *predictor* of coronary risk within populations. This is because a high proportion of individuals with high blood cholesterol concentrations have cardiovascular disease, yet a large proportion of individuals diagnosed with cardiovascular disease have concentrations within the normal range (Griffin 1999). Thus, while the interrelationships between diet and blood cholesterol may be of interest, it is impossible to conclude with absolute certainty the importance of the effects of dietary fatty acids on blood cholesterol with respect to cardiovascular disease in a normal population. This is not to say that dietary fat cannot influence atherosclerosis through other mechanisms. Some fatty acid classes (in particular the *n*-3 PUFAs) have profound effects on blood clotting and, potentially, thrombosis (British Nutrition Foundation 1992, 1999). Furthermore, fatty acids may directly affect the atherogenic process by modifying the susceptibility of LDL to oxidation, by modulating inflammatory functions of macrophages and by altering scavenger receptor expression and foam cell formation.

5.2
Dietary Fatty Acids and LDL Oxidation

Oxidative modification of LDL, which progressively degrades PUFA within the particle, increases its atherogenicity (Steinberg et al. 1989). The susceptibility of LDL to oxidation is influenced by its PUFA content (amount of substrate available for oxidation) and its antioxidant content (confer resistance to oxidation). Thus, at a given antioxidant content, decreasing the PUFA content of LDL should decrease its susceptibility to oxidation. Consumption of a diet rich in oleic acid, a MUFA, has been reported to decrease the susceptibility of LDL to oxidation ex vivo (Parthasarathy et al. 1990; Reaven et al. 1991; Berry et al. 1992), presumably because MUFAs replace PUFAs in LDL and are less susceptible to oxidation. Diets high in linoleic acid increase the linoleic acid content of LDL and in some studies this is associated with increased susceptibility to oxidation ex vivo (Parthasarathy et al. 1990; Abbey et al. 1993; Reaven et al. 1993;

Louheranta et al. 1996). However, some studies report no effect of increased linoleic acid consumption on susceptibility of LDL to oxidation (Nenseter et al. 1992; Suzukawa et al. 1995), although this may be due to the use of lower doses of linoleic acid than in some other studies. The effects of dietary *n*-3 PUFAs on LDL oxidation are unclear. Some studies demonstrate that fish oil supplementation of the diet increases the susceptibility of LDL to oxidation ex vivo (Suzukawa et al. 1995), presumably by increasing the PUFA content. However, other studies, using similar doses and similar measurements of lipid peroxidation, showed no effect of fish oil (Nenseter et al. 1992; Frankel et al. 1994). The discrepancies in the results may be related to the antioxidant content of the LDL, which was not reported in any of the studies and may differ. Thus, despite reported protective effects of fish oil against mortality from cardiovascular disease (Burr et al. 1989; Singh et al. 1997; Gissi Prevenzione 1999), it remains possible that *n*-3 PUFAs may, paradoxically, increase the susceptibility of LDL to oxidation.

5.3
Fatty Acids and the Atherogenicity of Oxidised LDL

The foam cell hypothesis, describing uptake of oxidatively modified LDL by macrophages, which subsequently become foam cells, is widely accepted (Kruth 2001). The foam cell hypothesis goes on to explain that the lipid core of an atherosclerotic plaque is derived from the release of cholesterol by dying foam cells. However, it is interesting to note that the fatty acid composition of cholesteryl esters from plaque regions containing foam cells is different from that in the lipid core region. Foam cells contain cholesteryl esters which are rich in oleic acid, whereas extracellular lipid particles in the lipid core have a high proportion of linoleic acid, similar in fact to the fatty acid profile of LDL cholesteryl esters (Smith and Slater 1972; Chao et al. 1990). Furthermore, a number of experimental studies suggest that subendothelial lipid accumulation precedes entry of monocytes into the subendothelial space and subsequent foam cell formation (Guyton and Kemp 1992). These observations suggest that there may be flaws in some aspects of the rationale for the foam cell hypothesis. It has been suggested as an alternative that the primary function of macrophages in atherosclerotic lesions may in fact be to *remove* cholesterol, since they can excrete the cholesterol they accumulate through many processes (Kruth 2001). Accordingly, pharmacological agents used to modulate foam cell formation can function to limit cholesterol uptake, to alter cholesterol esterification or trafficking within the macrophage (e.g. by modulating lysosomal degradation) or to enhance cholesterol efflux (Kruth 2001). Physiological agents, such as cytokines and hormones, may also modulate lipoprotein uptake and metabolism by macrophages. This suggests that there may be potential for the modulation of foam cell formation by dietary fatty acids, either through their actions on cytokines or through modifications in the fatty acid composition of LDL and/or macrophage lipids, resulting in altered uptake of oxidised LDL. However, this area has

been little studied to date. A few studies have examined the effects of *n*-3 PUFAs on scavenger receptor expression by monocytes or macrophages. An animal study demonstrated that feeding a fish-oil-rich diet to mice resulted in down-regulation of macrophage scavenger receptors AI and AII, while coconut oil and sunflower oil had no effect compared with the standard diet fed to the animals (Miles et al. 2000). Pietsch et al. (1995) reported a down-regulation of the expression of CD36 by the human monocytic U937 cell line after incubation with 5 μM EPA or DHA, but not with linoleic acid or AA. In another study, EPA (30–240 μM) was shown to inhibit the proliferation of the same cell line in a dose-dependent manner and, at the highest concentrations, induced apoptosis (Finstad et al. 1998). Expression of CD36 was lower in cells treated with 60 μM EPA or oleic acid compared with untreated cells (Finstad et al. 1998). However, EPA unexpectedly caused greater accumulation of lipid droplets in the cells than oleic acid, although the effects were reversed when cells were re-incubated in EPA-free medium. This leaves the question of the precise nature of the effects of fatty acids on foam cell formation unresolved. It is interesting to note, however, that the transcription factor, peroxisome proliferator activated receptor (PPAR)γ, has been reported to be involved in foam cell formation by virtue of its induction of CD36 (Nagy et al. 1998; Tontonoz et al. 1998). Since oxidised fatty acids are likely candidates as physiological ligands of PPARγ, it is possible that dietary modulation of the fatty acid composition of LDL could generate different patterns of oxidised derivatives, which could have differential effects on PPARγ.

Oxidation of LDL has many physiological effects that may influence atherosclerotic lesion development. In cell culture systems, foam cell formation by monocytic cells is rarely observed (Kruth 2001), reflecting a limitation in experimental techniques. These systems have been used, however, to investigate the pro-atherogenic and pro-inflammatory properties of oxidised LDL. Oxidised LDL is pro-atherogenic by virtue of its upregulated uptake by scavenger receptors. There has often been an assumption that oxidised LDL is pro-inflammatory in nature and that it is primarily the reaction of macrophages to oxidised LDL which confers the chronic inflammation characteristic of atherosclerosis. However, the evidence for this is rather limited. Oxidised LDL is reported to stimulate the expression of monocyte chemotactic protein, adhesion molecules and some cytokines (Berliner and Heinecke 1996). However, a number of studies report anti-inflammatory effects of oxidised LDL, including downregulation of CCR2 (Han et al. 2000) and downregulation of the platelet-activating factor receptor on monocytes (Hourton et al. 2001). There are even reports that oxidised LDL at low concentrations improves the viability of monocytes and that the purpose of this may be to maintain long-term survival of macrophages in lesions (Hamilton et al. 1999). Other studies demonstrate that oxidised LDL at high concentrations leads to apoptosis of vascular cells, which could contribute to plaque instability (Siow et al. 1999). Thus, the nature of the atherogenicity of oxidised LDL remains to be clarified. Since the fatty acid composition of LDL is

responsive to diet, it is possible that dietary modification could alter cellular responses to oxidised LDL, but this has not been studied to date.

5.4 Fatty Acids and Plaque Stability

The propensity of atherosclerotic plaques to rupture is influenced by their lipid content and the distribution of lipid within the plaque (Felton et al. 1997). There appears to be a reduction in the proportion of *n*-6 PUFAs and total PUFAs at the edges of disrupted plaques compared to the centres, which may reflect oxidative damage (Felton et al. 1997). It is therefore postulated that oxidised derivatives of PUFA may alter inflammatory activity and connective tissue degradation at the edges of lesions, enhancing the likelihood of disruption at this site (Felton et al. 1997). The effects of individual fatty acids and their oxidised derivatives have not been elucidated. However, given the evidence for the anti-coagulatory, anti-thrombotic and anti-inflammatory properties of *n*-3 PUFAs (British Nutrition Foundation 1992, 1999), it is possible that alteration of the PUFA composition of the diet could affect plaque progression, stability and thrombus formation. This has yet to be demonstrated in humans, but would strengthen the case for the reported protective effects of *n*-3 PUFAs in mortality from cardiovascular disease (Burr et al. 1989; Singh et al. 1997; Gissi Prevenzione 1999).

6 Summary of the Effects of Fatty Acids on Monocyte and Macrophage Functions

Dietary fatty acids, especially *n*-3 PUFAs, can modulate monocyte/macrophage activities. At high intakes *n*-3 PUFAs can affect chemotaxis, phagocytosis, respiratory burst, eicosanoid production, cytokine production and other monocyte/macrophage functions. Although some of the effects of *n*-3 PUFAs may be brought about by modulation of the amount and types of eicosanoids made, it appears that these fatty acids might elicit some of their effects by eicosanoid-independent mechanisms, including actions upon intracellular signalling pathways and transcription factor activity (see Miles and Calder 1998; Calder 2002). These effects of *n*-3 PUFAs are generally termed as "anti-inflammatory" and are considered to be beneficial to health (see Calder 2001a,b). The effects of dietary fatty acids on monocyte/macrophage function may also be relevant to atherosclerosis, which is now recognised to include an inflammatory component. Fatty acids could potentially affect the degree of oxidation of LDL, its uptake by vascular cells, aspects of foam cell formation and inflammatory activity within lesions.

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Macrophages as Therapeutic Targets in Lysosomal Storage Disorders

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Abstract Gaucher disease (lysosomal glucocerebrosidase deficiency) is a rare inborn error of metabolism. The type 1 variant is characterised by lysosomal storage of glucosylceramide in tissue macrophages exclusively. The accumulation of storage cells (Gaucher cells) results in pronounced hepatosplenomegaly, haematological abnormalities and deterioration of the skeleton. Type 1 Gaucher disease should be considered as a true macrophage disorder. Specific markers for Gaucher cells, like a hitherto unknown chitinase, have been identified and are commonly used to monitor progression of disease and efficacy of therapies. A spectacular correction in clinical symptoms of type 1 patients can be accomplished by chronic intravenous administration of human glucocerebrosidase containing glycans with terminal mannose-moieties. Currently, about 3,000 patients are treated worldwide with recombinant enzyme (Cerezyme). Enzyme replacement therapy (ERT) is not able to prevent glucosylceramide accumulation in the brain of patients suffering from the severe type 2 variant of Gaucher disease. Recently, oral administration of *N*-butyl-deoxynojirimycin has been registered in the EU for treatment of type 1 Gaucher patients that are unsuitable for ERT. The iminosugar inhibits the synthesis of glucosylceramide and thus prevents massive accumulation of the lipid.

Keywords Chitinase, Deoxynojirimycin, Enzyme replacement, Gaucher disease, Glucocerebrosidase, Glucosylceramide, Lysosome, Substrate reduction

1 Introduction to Lysosomes and Lysosomal Storage Disorders

1.1 Lysosomes

The continuous recycling of their macromolecular constituents is a hallmark of the long-lived eukaryotic cell. For this reason mammalian cells contain single membrane-enclosed compartments in which a variety of biological macromolecules can be safely and efficiently degraded. Based on their lytic function, these acid organelles have been named lysosomes (De Duve et al. 1955). Substrates for lysosomal degradation can enter the organelles via different routes, such as endocytosis, pinocytosis, phagocytosis and autophagocytosis. In addition, direct chaperon-mediated import of specific proteins from the cytoplasm has been reported (Holzmann 1989; Dice et al. 1990). Lysosomes are equipped with a set of about 60 acid hydrolases and a dozen accessory proteins that allow sequential degradation of almost all natural macromolecules, including lipids, glycosaminoglycans, oligosaccharides, proteins and nucleic acids. Mediated by specific carriers in the lysosomal membrane, the products of intralysosomal catabolism are exported to the cytoplasm where they can be re-utilised. The lysosomal membrane is effectively protected against self-digestion by the presence of transmembrane proteins with large, highly glycosylated intralysosomal domains (Peters and von Figura 1994).

The prominent lectin-based mechanism that governs the selective routing of newly formed acid hydrolases to lysosomes was elucidated two decades ago (Kornfeld and Mellman 1989). Upon co-translational translocation of lysosomal enzymes into the lumen of the endoplasmic reticulum, their signal peptide is removed and specific asparagine residues are glycosylated by transfer of a preformed oligosaccharide from a dolichol phosphate lipid carrier. The glycoproteins are folded, assembled in correct multimeric structures, and terminal glucose moieties are removed from their glycans, an important checkpoint in the quality control of protein folding (Helenius 1994). Next, the glycoproteins are exported to the Golgi apparatus where some of their oligosaccharide chains exclusively obtain mannose-6-phosphate moieties by a two-step process. The phosphomannosyl moieties act as a specific recognition signal. Selective binding of a major fraction of most lysosomal enzymes to cation-dependent or cation-independent mannose-6-phosphate receptors (MPRs), allows their segregation from the secretory proteins in the trans-Golgi network. In endosomal compartments, dissociation of mannose-6-phosphate receptors and lysosomal protein ligands occurs due to local acidity. Following uncoupling, the receptor recycles to the Golgi apparatus and the newly formed hydrolases are delivered into

lysosomes. The cation-independent MPR is also involved in the delivery to lysosomes of extracellular soluble acid hydrolases containing mannose-6-phosphate residues. In contrast, targeting of integral lysosomal membrane proteins is not mediated by phosphomannosyl moieties but by specific motifs in their cytoplasmic domains. Further alternative targeting mechanism to lysosomes have to exist. Some membrane-associated lysosomal enzymes like glucocerebrosidase do not acquire phosphomannosyl moieties at all in their glycans but are nevertheless efficiently targeted to lysosomes by still unknown mechanisms (Aerts et al. 1988). The lysosomal targeting of lysozyme and chitotriosidase in macrophages is also independent of lectin receptors, since these enzymes completely lack N-linked glycans (Renkema et al. 1997). Moreover, investigations on patients suffering from I-cell disease, in which formation of phosphomannosyl moieties is impaired, have indicated that in hepatocytes and lymphocytes very efficient intracellular sorting of newly formed soluble acid hydrolases can also occur independently of mannose-6-phosphate receptors (Owada and Neufeld 1982). The precise mechanism of the mannose-6-phosphate independent targeting of soluble acid hydrolases is yet unknown, but it has been suggested that it involves a transient membrane association in the Golgi apparatus (Rijnboutt et al. 1991).

1.2 Lysosomal Storage Disorders

The physiological importance of the degradative processes in lysosomes is revealed by the existence of a group of at least 40 distinct inherited diseases, the so-called lysosomal storage disorders (Neufeld 1991; Gieselmann 1995). Most of these diseases are caused by a deficiency in a single lysosomal enzyme or essential cofactor and result in the lysosomal accumulation of one or sometimes several natural compounds. According to the prevailing stored compound, the lysosomal storage diseases are grouped as mucopolysaccharidoses, sphingolipidoses, mucolipidoses, lipidoses, glycoproteinoses, glycogenosis and ceroid lipofuscinoses. Some lysosomal storage disorders are not single enzymopathies but based on defects in transport of hydrolytic products across the lysosomal membrane, deficiencies in non-lysosomal proteins involved in lysosome biogenesis or post-translational modification of lysosomal enzymes or inherited abnormalities in intracellular membrane flow.

All lysosomal storage diseases are relatively rare with an overall incidence for the whole group of 1:5,000–1:10,000. The individual incidence of the more prominent lysosomal diseases is between 1:20,000 and 1:100,000 in most populations (Meikle et al. 1999; Poorthuis et al. 1999). Genetic drift and founder effects have led to unusually high incidences of specific lysosomal storage diseases in some populations. The best examples of this are Gaucher and Tay-Sachs disease among Ashkenazim, and aspartylglucosaminuria, Salla disease and infantile neuronal ceroid lipofuscinosis in Finland (Peltonen 1997). As an example, Table 1 summarises the nature and prevalence of one subgroup of the lysosomal storage disorders: the sphingolipidoses.

Table1 Birth prevalence (per 100,000)of sphingolipidoses in The Netherlands

Disease	Prevalence
Fabry	0.21
Gaucher	1.16
Niemann-Pick type A and B	0.53
Niemann-Pick type C	0.35
Krabbe	1.35
Sandhoff	0.34
Tay-Sachs	0.41
GM1-gangliosidosis	0.41

The clinical manifestation of lysosomal storage disorders is remarkably heterogeneous, contributing to the limited awareness of these diseases. Age of onset and progression of disease vary considerably for almost each individual storage disorder. This remarkable phenotypic variability is usually linked to the extent of the deficiency that is determined by the exact nature of the underlying genetic defect. In the case of some lysosomal enzymopathies, a strict correlation between residual enzyme activity and severity of disease manifestation exists. A common feature of lysosomal storage disorders is that accumulation of storage material is generally restricted to lysosomes of particular cell types. The nature and residual capacity of the defective metabolic pathway in combination with the actual flux through this pathway in various cell types determine the chance that particular cell types are affected. This phenomenon explains why in some lysosomal storage disorders, external genetic or environmental factors that influence the flux through the defective pathway also have a major impact on disease manifestation. The genotype–phenotype relation is therefore not strict in many lysosomal storage disorders.

The lysosomal apparatus of tissue macrophages fulfils many important degradative functions. Macrophages participate in the degradation of invading microbes, the natural turnover of blood cells and tissue modelling. In view of this, it is not surprising that in a considerable number of the lysosomal storage disorders accumulation of storage material also takes place prominently in tissue macrophages. The relatively common type 1 variant of Gaucher disease is unique with respect to the fact that lysosomal storage occurs exclusively in macrophages. The remainder of this review will deal with Gaucher disease and the progress that has been made regarding therapeutic correction of this macrophage disorder.

2 Gaucher Disease: A Macrophage Disorder

Gaucher disease is the most frequently encountered lysosomal storage disorder in man (Barranger and Ginns 1989; Beutler and Grabowski 1995). In 1882 the clinical features of the disease were first described in detail by the French medi-

cal student Philippe C.E. Gaucher, reporting the presence of large unusual cells in a 32-year-old female with an enlarged spleen. Already at the beginning of the last century it was suggested that the disease was a familial disorder. In 1934 the primary storage material in Gaucher disease was finally identified as glucocerebroside (glucosylceramide). The glycosphingolipid glucocerebroside is the common intermediate in the synthesis and degradation of gangliosides and globosides. In 1965 Patrick and Brady et al. independently showed that the primary defect in Gaucher disease is a marked deficiency in activity of the lysosomal enzyme glucocerebrosidase (EC. 3.2.1.45) (Brady et al. 1965; Patrick 1965). Inherited deficiencies in glucocerebrosidase result in accumulation of its lipid substrate in the lysosomal compartment of macrophages throughout the body. Three different phenotypes are recognised, which are differentiated on the basis of the presence or absence of neurological symptoms. The most prevalent variant of the disease is the non-neuronopathic form, named type 1 Gaucher disease. The age of onset and clinical manifestations of type 1 Gaucher disease are highly variable. The most common symptoms include splenomegaly with anaemia and thrombocytopaenia, mostly due to hypersplenism, hepatomegaly and bone disease. Anaemia may contribute to chronic fatigue. Thrombocytopaenia and prolonged clotting times may lead to an increase in bleeding tendency. Atypical bone pain, pathological fractures, avascular necrosis and extremely painful bone crises may also have a great impact on the quality of life. Type 1 Gaucher disease is relatively common in all ethnic groups. It is prevalent among Ashkenazim with a carrier frequency as high as about 1 in 10 and an incidence of about 1 in 450. The most common mutation in the glucocerebrosidase gene of Caucasians, including Ashkenazim, encodes the amino acid substitution N370S. The heteroallelic presence of the N370S mutation is always associated with a non-neuronopathic course (Jonsson et al. 1987). It has been demonstrated that the N370S glucocerebrosidase is normally produced and present in lysosomes. Its catalytic activity is only severely impaired at pH values above 5.0, illustrating the subtle nature of the mutation (Van Weely et al. 1993). Most, but not all, homozygotes for the N370S mutation do not develop significant clinical symptoms. Twin studies and the poor predictive power of phenotype–genotype investigations in Gaucher disease have clearly pointed out that epigenetic factors also play a key role in Gaucher disease manifestation (Aerts et al. 1993; Cox and Schofield 1997).

Although glucocerebrosidase is present in lysosomes of all cell types, type 1 Gaucher disease patients develop storage of glucocerebroside solely in cells of the mononuclear phagocyte system. It is believed that the storage material stems from the breakdown of exogenous lipids derived from the turnover of blood cells. The glucocerebroside-loaded cells show a characteristic morphology with a 'wrinkled paper'-like appearance of their cytoplasm, which contains lysosomal inclusion bodies; these cells are referred to as Gaucher cells. In the last decades it has become apparent that Gaucher cells are not inert containers of storage material but viable, chronically activated macrophages that secrete various factors that contribute to the diverse clinical manifestations of Gaucher dis-

ease. Increased circulating levels of several pro-inflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8], the anti-inflammatory cytokine IL-10, and macrophage colony-stimulating factor (M-CSF) have been reported (Aerts and Hollak 1997; Cox 2001). It has been hypothesised that cytokine abnormalities may play a crucial role in the development of common clinical abnormalities in Gaucher patients such as osteopaenia, activation of coagulation, hypermetabolism, gammopathies and multiple myeloma and hypolipoproteinaemias. More recently, examination of gene expression profiles by suppressive subtraction hybridisation analysis of Gaucher and control spleens has led to the identification of over-expression by Gaucher cells of transcripts for cathepsins B, K and S (Moran et al. 2000). It is of interest to note that osteoclast-derived cathepsin K is prominently involved in osseous type I collagen destruction. Local release of this cathepsin by Gaucher cells may contribute to the osteolysis in Gaucher disease.

3
Therapy of Type 1 Gaucher Disease

Type 1 Gaucher disease has generally been considered to be the most attractive candidate among the inherited lysosomal storage disorders for developing effective therapeutic interventions. First, the molecular basis of the underlying genetic defect had been already established in detail at the gene and protein level. Second, just a single cell type, the tissue macrophage, is primarily implicated in the pathophysiology of the disorder. The rationale for therapeutic intervention of type 1 Gaucher disease is therefore relatively simple: correction (or prevention of ongoing formation) of Gaucher cells. This could be accomplished by supplementation of macrophages with the enzyme glucocerebrosidase (enzyme replacement therapy), by reduction of glycolipid synthesis with specific inhibitors (substrate deprivation or substrate balancing therapy), or by introduction of glucocerebrosidase cDNA in haematopoietic progenitors of macrophages (gene therapy).

3.1
Enzyme Therapy

Thanks to the pioneering work of Brady, Barranger and co-workers at the National Institutes of Health (Bethesda, USA) as well as valuable contributions by many others, a highly effective treatment of type 1 Gaucher disease is now feasible based on chronic intravenous administration of human glucocerebrosidase (Brady 1997; Barranger and O'Rourke 2001). The first attempts to treat type 1 Gaucher disease by infusions with glucocerebrosidase isolated from human placenta were already started in the early 1970s at the National Institutes of Health. Unfortunately, these did not result in an effective therapy for two compelling reasons. In the first place, too little and insufficiently pure glucocerebrosidase could be generated with the existing technology. In the second place, most of

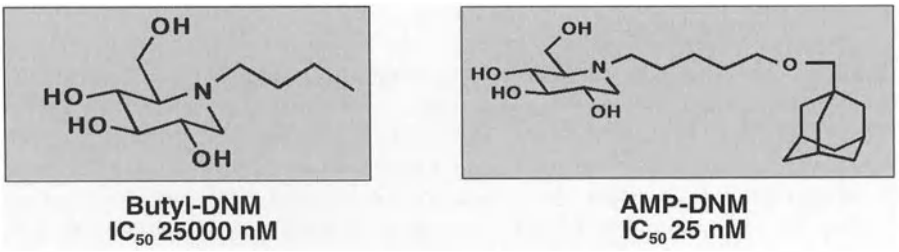

Fig. 1 Structures of deoxynojirimycin-type inhibitors of glycosylceramide synthase. *Butyl-DNM*, butyl-deoxynojirimycin; *AMP-DNM*, adamantane-pentyl-deoxynojirimycin

the administered enzyme was not delivered to macrophages but to other cell types such as hepatocytes. The final development of an effective enzyme replacement therapy for type 1 Gaucher disease relied on a fortunate intersection of scientific disciplines: the discovery of receptors for glycoproteins and the complete purification of glucocerebrosidase. Purification of the protein to homogeneity was achieved in 1977 and subsequently isolation procedures were markedly improved (Murray et al. 1985; Aerts et al. 1986). In 1974, the first mammalian cell lectin, the asialoglycoprotein receptor, was described and next a mannose-specific lectin on Kupffer cells in the liver was identified (Ashwell and Morell 1974). The mannose receptor was shown to interact avidly with mannose-terminal glycoconjugates and mediate their delivery into lysosomes (Stahl et al. 1978). It was realised by Barranger, Brady and co-workers that this receptor-mediated uptake mechanism could be exploited for therapy of Gaucher disease. Analysis of the carbohydrate composition of placental glucocerebrosidase showed the presence of three complex-type glycans and a single high mannose-type glycan per molecule (Takasaki 1984). The presence of terminal galactose moieties in the glycans of placental glucocerebrosidase provided an explanation for its undesired preferential targeting to hepatocytes. To increase the amount of terminal mannose moieties in placental glucocerebrosidase, an in vitro method based on sequential enzymatic removal of *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine moieties with exoglycosidases was developed (Furbish et al. 1981) (see Fig. 1). The modified 'mannose-terminated' glucocerebrosidase remained fully enzymatically active. It has been further demonstrated that a similar mannose-terminated form of the enzyme is generated by sequential action of lysosomal exoglycosidases during maturation of endogenous glucocerebrosidase in lysosomes of human fibroblasts (Van Weely et al. 1990). Animal studies with the mannose-terminated placental glucocerebrosidase revealed that the enzyme was delivered differentially to Kupffer cells in comparison with hepatocytes (Furbish et al. 1981). Upon treating a 5-year-old Ashkenazi Jewish boy with the modified placental enzyme, Barranger and co-workers noted promising clinical improvements. In subsequent years the involvement of industry (Genzyme Corporation, Boston, USA) was required to produce sufficient enzyme for further clinical studies with mannose-terminated

placental glucocerebrosidase (Ceredase). In 1990, Barton and co-workers finally demonstrated unequivocally in a study with 12 type 1 Gaucher patients that twice-weekly intravenous administration of Ceredase (130 IU/kg/month) resulted in marked improvement in organomegaly and corrections of haematological abnormalities (Barton et al. 1991). The spectacular clinical response to enzyme replacement therapy has led to a rapid application worldwide. At present close to 3,000 type 1 Gaucher patients benefit from therapeutic intervention with Cerezyme, the recombinant form of glucocerebrosidase that has superseded the placenta-derived Ceredase (Grabowski et al. 1995).

The introduction of Ceredase was associated with considerable controversy regarding optimal dosing regimens, further stimulated by concerns regarding the safety of the incompletely pure placental enzyme preparation and the extreme costs for treatment of adult patients (US $50,000–$500,000 per patient per year). The availability of pure recombinant glucocerebrosidase and clinical investigations on optimal individualised dosing regimens have resolved most of the debate (Hollak et al. 1995). However, still at this moment little is known about the true efficacy of targeting of mannose-terminated glucocerebrosidase to macrophages or Gaucher cells. Investigations in rats have revealed that a major fraction of Ceredase is not delivered to macrophages but endocytosed by liver endothelial cells (Bijsterbosch et al. 1996). This is not unexpected, since it had been earlier demonstrated that the mannose-receptor is also expressed on these cells (see Linehan et al. 1999). Although elegant studies with radiolabelled enzyme in volunteers have been conducted by Mistry, it remains an unanswered question to which cells precisely mannose-terminated glucocerebrosidase is delivered in Gaucher patients (Mistry et al. 1996).

Systemically administered glucocerebrosidase, a glycoprotein of about 52 kDa, is unable to pass the blood–brain barrier. The outcome of enzyme replacement therapy for acute neuronopathic (type 2) and severe forms of chronic neuronopathic (type 3) Gaucher disease is disappointing (Erikson 2001). Several clinical investigations have revealed that in the severe neuronopathic Gaucher patients, the effects of enzyme replacement therapy on visceral and haematological symptoms are good, but the fatal neurological deterioration continues. Accumulation of glucocerebroside and its metabolite glucosylsphingosine inside the brain underlies the severe neuropathology of these patients. Importantly, milder forms of type 3 Gaucher disease, where the chronic neuronopathic disease is primarily caused by perivascular storage cells, respond well to enzyme replacement therapy; and treatment with a high-dose enzyme regimen is recommended by the European Working Group on Gaucher Disease (Vellodi et al. 2001). Perivascular macrophages in the brain are known to express mannose receptor (Linehan et al. 1999).

3.2 Substrate Deprivation Therapy

An alternative approach for therapeutic intervention of type 1 Gaucher and other glycosphingolipidoses is substrate deprivation (also termed substrate reduction) therapy. Radin and coworkers firstly formulated the challenging concept (see for a review Radin 1996). The approach aims to reduce the rate of glycosphingolipid biosynthesis to levels which match the impaired catabolism. It is conceived that patients who have a significant residual lysosomal enzyme activity could gradually clear lysosomal storage material and therefore should profit most from reduction of substrate biosynthesis.

Two main classes of inhibitors of glycosphingolipid biosynthesis have been described, both of which inhibit the ceramide-specific glucosyltransferase (also termed glucosylceramide synthase; GlcT-1; UDP-glucose: *N*-acylsphingosine d-glucosyl-transferase, EC 2.4.1.80). The enzyme catalyses the transfer of glucose to ceramide, the first step of the biosynthesis of glucosphingolipids. The first class of inhibitors is formed by analogues of ceramide. The prototype inhibitor is PDMP (d, l-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol). More specific and potent analogues have been subsequently developed based on substituting the morpholino group for a pyrrolidino function and by substitutions at the phenyl group: 4-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidono-1-propanol (p-OH-P4) and ethylenedioxy-1-phenyl-palmitoylamino-3-pyrrolidino-1-propanol (EtDo-P4) (Lee et al. 1999). Studies in a knock-out mouse model for Fabry disease have shown that oral administration of the compounds can result in a marked reduction of the accumulating glycosphingolipid globotriaosylceramide (Abe et al. 2000). The second class of inhibitors of glucosylceramide synthase is formed by *N*-alkylated iminosugars. Such compounds were already in common use as inhibitors of N-glycan processing enzymes, and the potential application of *N*-butyl-deoxynojirimycin (NB-DNJ) as HIV inhibitor had been studied in AIDS patients. Platt and Butters at the Glycobiology Institute in Oxford were the first to recognise the ability of NB-DNJ to inhibit glycosylceramide synthesis at low micromolar concentrations (Platt and Butters 1995). The same researchers demonstrated in knock-out mouse models of Tay-Sachs disease and Sandhoff disease significant reductions in glycosphingolipid storage in the brain (Jeyakumar et al. 1999). Preclinical studies in animals and the previous clinical trial in AIDS patients have indicated (transient) adverse effects in the gastrointestinal tract, probably related to the ability of NB-DNJ to inhibit disaccharidases on the intestinal brush border. Animal studies have shown that the galactose analogue *N*-butyl-deoxygalactonojirimycin (NB-DGJ) may have the same therapeutic efficacy as NB-DNJ but does not cause gastrointestinal side-effects (Andersson et al. 2000). Overkleeft and coworkers in their search for inhibitors of glucosidases have serendipitously developed a more potent inhibitor of glucosylceramide synthase. Adamantane-pentyl-deoxynojirimycin (AMP-DNM) was found to inhibit glycosphingolipid biosynthesis at low nanomolar concentrations (Overkleeft et al. 1998) and able to prevent globo-

triaosylceramide accumulation in a Fabry knock-out mouse model without overt side-effects (D. Copeland, personal communication).

The first clinical study of the use of NB-DNJ to treat a glycosphingolipid storage disorder was reported recently (Cox et al. 2000). In an open-label phase I/II trial, 28 adult type 1 Gaucher patients received three times daily 100 mg NB-DNJ (OGT918; Oxford GlycoSciences). Improvements in visceromegaly and haematological abnormalities as well as corrections in plasma levels of glucosylceramide and biomarkers of Gaucher disease activity have been described, although the extent of the response is less spectacular than generally observed with high-dose enzyme replacement therapy. Provided that iminosugars or other inhibitors of glucosylceramide synthase prove to be safe in the long term, they will have an important role to play in the management of glycosphingolipid storage disorders, including Gaucher disease.

3.3 Gene Therapy

Since tissue macrophages are derived from bone marrow, it is logical that curative bone marrow transplantations have been reported for some patients with Gaucher disease (Ringden et al. 1995). The risks of allogeneic transplantation, however, do not justify this approach in patients with milder forms of the disease. The observed efficacy of enzyme replacement therapy and bone marrow transplantation has stimulated the pursuit of gene therapy for Gaucher disease. Three independent studies of gene transfer to the haematopoietic cells of Gaucher patients have been conducted but none produced encouraging results (Richter and Karlsson 2001). Low transduction efficiencies of CD34 cells and no sustained expression of glucocerebrosidase in white blood cells have contributed to this. The development of gene therapy strategies to correct haematological and genetic disorders has been hampered by the low levels of gene transfer into human stem cells using vectors derived from oncoretroviruses. Much interest has been recently focused on vectors derived from lentiviruses that have been shown to transduce a variety of nondividing cells, including haematopoietic cells (Richter and Karlsson 2001). The use of such vectors and new developments with respect to macrophage-specific gene targeting (see Chap. 6, this volume) may open novel possibilities for effective gene therapy of Gaucher disease in the future.

4 Monitoring of Therapeutic Correction

Considerable attention has been paid in relation to type 1 Gaucher disease to treatment goals and the monitoring of response to therapeutic interventions (Cox 2001; Hollak et al. 2001). The definition of treatment goals has to depend on clinical endpoints or surrogate endpoints that can predict clinical benefit based on epidemiological, pathophysiological or other scientific evidence. In

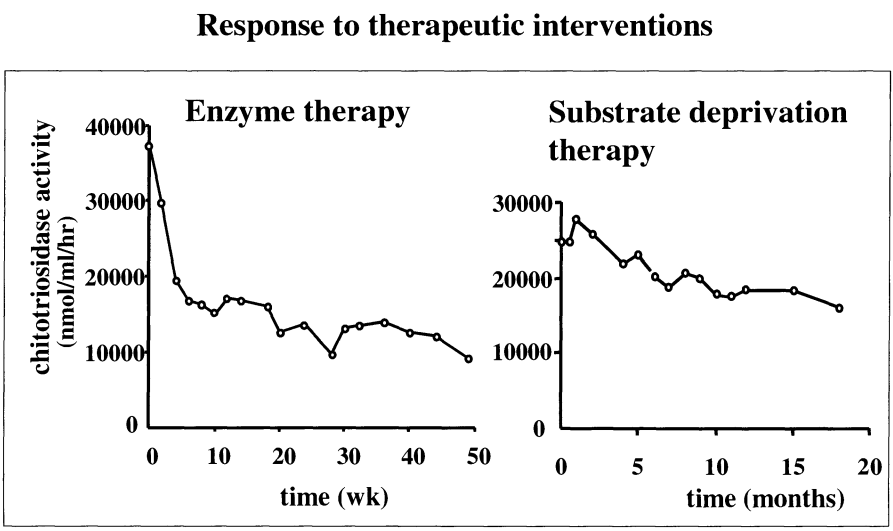

Fig. 2 Corrections in elevated plasma chitotriosidase following therapeutic intervention. *Left panel*: Response of the first type 1 Gaucher patient treated in continental Europe by intravenous administration of Ceredase (48 IU/kg/month). *Right panel*: Response of first type 1 Gaucher disease patient treated in continental Europe by oral administration of butyl-deoxynojirimycin (300 mg/day)

view of the burden imposed by chronic intravenous infusions and the high costs associated with enzyme therapy as well as the uncertainty regarding dose-dependent, long-term adverse effects of iminosugar therapy, it seems wise to establish for the individual Gaucher patient the minimal dose of drug required for effective intervention. In severely affected patients, the initial response to therapy can be accurately assessed by determination of spleen and liver volumes, haemoglobin level and platelet count. During maintenance therapy, however, these clinical parameters are of little value. Monitoring the effect of therapy on bone disease is complicated and has usually been restricted to documentation of the occurrence of bone crises, pathological fractures or the need for surgical intervention. More recently, quantitative chemical shift imaging (QCSI) has been applied to study the triglyceride content of lumbar bone marrow (Hollak et al. 2001). The fat fraction of the bone marrow is variably reduced in Gaucher disease due to displacement of normal triglyceride-rich adipocytes by Gaucher cells. It has been noted that a marked reduction in bone marrow fat fraction is predictive for the occurrence of bone complications. A marked correction in bone marrow fat content following therapy can be therefore defined as a treatment goal.

A search for plasma abnormalities in Gaucher disease has led to the discovery of a marked elevation in chitotriosidase, a hitherto unknown human chitinase (Hollak et al. 1994). In symptomatic Gaucher patients, plasma chitotriosidase levels were found to be about 1,000-fold higher than in normal individuals. It has been subsequently shown that Gaucher cells are the source of this hydrolase

in plasma and that the elevated levels are an indicator of the burden of storage cells in a patient. Chitotriosidase is synthesised in the pathological macrophages, and its elevated activity correlates with tissue glucosylceramide storage as well as clinical parameters of disease severity. Enzyme replacement therapy, substrate deprivation therapy or bone marrow transplantation rapidly reduces the plasma chitotriosidase activity (see Fig. 2). To assess the utility of chitotriosidase activity measurements as a biomarker for treatment efficacy, the relationship between and clinical parameters has been studied (Hollak et al. 2001). On the basis of this investigation, it has been proposed that in patients in whom initiation of treatment is questionable, based solely on clinical parameters, a chitotriosidase activity above 15,000 nmol/ml/h may serve as an indicator of a high Gaucher cell burden and an indication for the initiation of treatment. A reduction of less than 15% after 1 year of treatment should be a reason to consider a dose increase. Furthermore, a sustained increase in chitotriosidase at any point during treatment should alert the physician to the possibility of clinical deterioration and the need for dose adjustment. The assay of chitotriosidase activity is complicated by the existence of apparent substrate inhibition due to transglycosidase activity (J.M. Aerts, manuscript in preparation). Another pitfall results from the complete absence of the enzymatic activity in about 6% of all individuals. This results from homozygosity for a null allele of the chitotriosidase gene (Boot et al. 1998). Plasma chitotriosidase levels in heterozygotes for this mutation (about 35% of all individuals) can lead to an underestimation of the actual presence of Gaucher cells in patients. Determination of chitotriosidase genotype in Gaucher patients is therefore recommended.

Chitotriosidase has been characterised in detail at the gene and protein level (Boot et al. 1995, 1998; Renkema et al. 1995, 1997). The enzyme mimics lysozyme in several aspects. It is also selectively expressed in phagocytes, particularly in chronically activated macrophages, and likewise is a compact globular endoglucosaminidase lacking N-linked glycans. The physiological role of chitotriosidase seems to be found also in innate immunity. It has been observed in studies with *Candida albicans* and *Aspergillus fumigatus* that the enzyme exerts a potent fungistatic effect by selective lysis of the growth tip of hyphae. The molecular basis for the massive overexpression of chitotriosidase in Gaucher cells and in related foam cells observed in arteriosclerosis, sarcoidosis, Wolman disease and Niemann-Pick disease is still unknown and the subject of ongoing investigation.

5 Prospects

In the last decade enormous progress has been made in therapy of type 1 Gaucher disease, a severely debilitating disorder characterised by intralysosomal storage of glucocerebroside in tissue macrophages. A highly effective therapy based on chronic intravenous administration of mannose-terminated recombinant human glucocerebrosidase is available. During the past decade this therapy

has been applied in several thousand patients without serious adverse effect. Moreover, for the same orphan disease, promising clinical responses have been observed upon oral administration of an iminosugar inhibitor of glucosylceramide synthesis. Provided that long-term treatment with such inhibitors is without adverse effects, substrate deprivation therapy (in conjunction with enzyme replacement therapy) may play an important role in the future clinical management of patients suffering from glycosphingolipid storage disorders. Progress in vector technology and selective expression of the transgene in macrophages seem to be essential requirements before gene therapy can fulfil its promise as cure for type 1 Gaucher disease.

Despite the success of the present enzyme replacement therapy with Cerezyme, the question should be raised whether the enzyme supplementation treatment can be further improved in order to be more economic and widely available. For example, it is unclear which percentage of the mannose-terminated Cerezyme is actually endocytosed by tissue macrophages and storage cells and which percentage is 'wasted' in other cell types such as liver endothelial cells. The occurrence and consequences of binding of the therapeutic enzyme to receptors other than the mannose receptor, to soluble receptor fragments like soluble mannose receptor (sMR), or to serum mannose-binding lectins still warrants further examination. Little attention has so far been paid to the expression of the mannose receptor on macrophages and other cells types of Gaucher patients. Increased knowledge about this matter may give valuable clues for further improvement of the current enzyme replacement therapy. Similar considerations can be made with respect to substrate deprivation therapy. In the case of type 1 Gaucher disease, one would prefer to inhibit selectively the synthesis of glucosylceramide in blood cells. More selective targeting of drugs to blood cells might therefore result in major improvement of efficacy and reduce the risk for side-effects.

It seems likely that Gaucher disease will also serve in the future as an interesting and challenging model for developing new or improved therapy modalities for the correction of lipid-laden tissue macrophages.

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Nuclear Receptors as Regulators of Macrophage Homeostasis and Function

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Abstract Nuclear hormone receptors comprise a superfamily of ligand-dependent transcription factors that regulate diverse aspects of development and homeostasis. Several members of this superfamily play important roles in the regulation of inflammatory responses and lipid homeostasis in macrophages. These include the glucocorticoid receptor, which acts to inhibit inflammatory programs of gene expression in response to natural corticosteroids and synthetic anti-inflammatory agents such as dexamethasone, peroxisome proliferator-activated receptors (PPARs) that regulate fatty acid homeostasis and inflammation in response to endogenous eicosanoids, and liver X receptors (LXRs) that regulate cholesterol efflux in response to endogenous oxysterols. Recent progress in defining the physiological roles of these receptor systems in macrophages and understanding their mechanisms of action suggest that they may be important targets for the development of new classes of pharmaceuticals that will be useful for treating human diseases in which macrophages play critical pathogenic roles, such as atherosclerosis and arthritis.

Keywords Atherosclerosis, Cholesterol, Foam cell, Glucocorticoid receptor, Inflammation, Liver X receptor, NF?B, Nuclear receptor, Peroxisome proliferator-activated receptor, Transcription, Vitamin D receptor

1 Introduction

Nuclear receptors comprise a superfamily of ligand-dependent transcription factors that regulate diverse aspects of development, homeostasis, and immune function (Evans 1988; Kastner et al. 1995; Mangelsdorf and Evans 1995; Chawla et al. 2001). Forty-eight distinct genes encoding nuclear receptors have been identified in humans and mice (Maglich et al. 2001). Several members of the nuclear receptor superfamily have been shown to play important physiologic roles in macrophages. This chapter will focus on the most extensively characterized of these: the glucocorticoid receptor α (GRα), estrogen receptor α (ERα), vitamin D receptor (VDR), retinoid X receptor α (RXRα), peroxisome proliferator-activated receptors α and γ (PPARα and γ), and the liver X receptors α and β (LXRα and LXRβ). Each of these receptors are regulated by small molecular weight ligands and are well-established or emerging targets of drugs that are used to treat human diseases in which macrophages play prominent pathogenic roles. Recent advances in the understanding of nuclear receptor biology and function raise the prospect that new generations of nuclear receptor ligands can be developed with improved therapeutic activities.

1.1 Domain Structure

Nuclear receptors share conserved modular domains that are illustrated in Fig. 1. These include a variable N-terminal activation domain (AF1), a highly conserved DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Evans 1988). The DBD consists of two interdependent zinc fingers that mediate specific DNA binding of nuclear receptor monomers, dimers, and heterodimers to hormone response elements (HREs) in direct target genes. Crystal structures of several nuclear receptor DBDs bound to their respective HREs indicate that the DBD provides both specific and non-specific DNA interactions that allow for general DNA binding and recognition of receptor-specific sequences (Luisi et al. 1991; Rastinejad et al. 1995). Most hormone response elements contain two or more closely spaced core recognition motifs, each of which is contacted by a single DBD. Steroid hormone receptors recognize palindromic inverted repeats as homodimers. An important subset of nuclear receptors that includes the retinoic acid receptors, PPARs and LXRs bind to DNA as heterodimers with a common partner, RXR (Yu et al. 1991; Kliewer et al. 1992; Chawla et al. 2001). In contrast to steroid hormone receptors, RXR heterodimers generally bind to target genes in the presence or absence of ligand. In

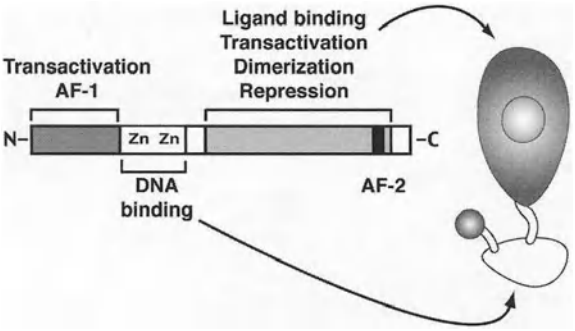

Fig. 1 Domain structure of nuclear receptors. Nuclear receptors exhibit a common modular domain structure. The N-terminus encodes a variable activation domain (*AF-1*) that is particularly important for the function of steroid hormone receptors. The DNA-binding domain is highly conserved and mediates specific interaction with a 6-bp core recognition sequence in hormone response elements. The ligand-binding domain determines the specific ligand-binding properties of each nuclear receptor and undergoes ligand-dependent conformational changes that control coactivator, corepressor, and heat shock protein interactions

addition, RXR heterodimers prefer HREs that consist of direct repeat organizations of the core recognition motifs rather than the palindromic HREs recognized by steroid hormone receptors (Umesono and Evans 1989; Näär et al. 1991; Umesono et al. 1991). Protein–protein interaction surfaces within the DBD determine the optimum spacing and orientation of core recognition motifs for each nuclear receptor dimer or heterodimer, and thereby play important roles in determining specificity of HRE recognition (Rastinejad et al. 1995).

The ligand-binding domain integrates several nuclear receptor functions. In addition to binding hormone, the LBD plays roles in dimerization/heterodimerization, subcellular localization, and ligand-dependent transcriptional activation and repression. In the case of steroid hormone receptors, the LBD also mediates interactions of unliganded receptors with heat shock protein complexes (Fig. 2). Hormone binding results in dissociation of steroid receptors from these complexes and acquisition of transcriptional activity (Evans 1988). Within the nucleus, the major transcriptional role of the LBD is to mediate interactions with coactivator and corepressor proteins in a ligand-dependent manner. This is thought to occur primarily through ligand-dependent conformational changes in an alpha helical region in the C-terminus referred to as activation function 2 (AF2). Crystal structures of a number of nuclear receptor LBDs in the absence and presence of cognate ligands suggest that ligand binding causes the AF2 helix to go from an extended or relatively mobile position to an "active" position, in which it is tightly bound to the LBD and in some cases interacts directly with the ligand (Bourguet et al. 1995; Renaud et al. 1995; Wagner et al. 1995; Moras and Gronemeyer 1998). This structural shift creates a "charge clamp" that interacts with a short helical motif in nuclear receptor coactivators that contains the consensus LxxLL, where L is leucine and x is any amino acid (Heery et al. 1997; Torchia et al. 1997). Because the position of the AF2 domain relative to the LBD

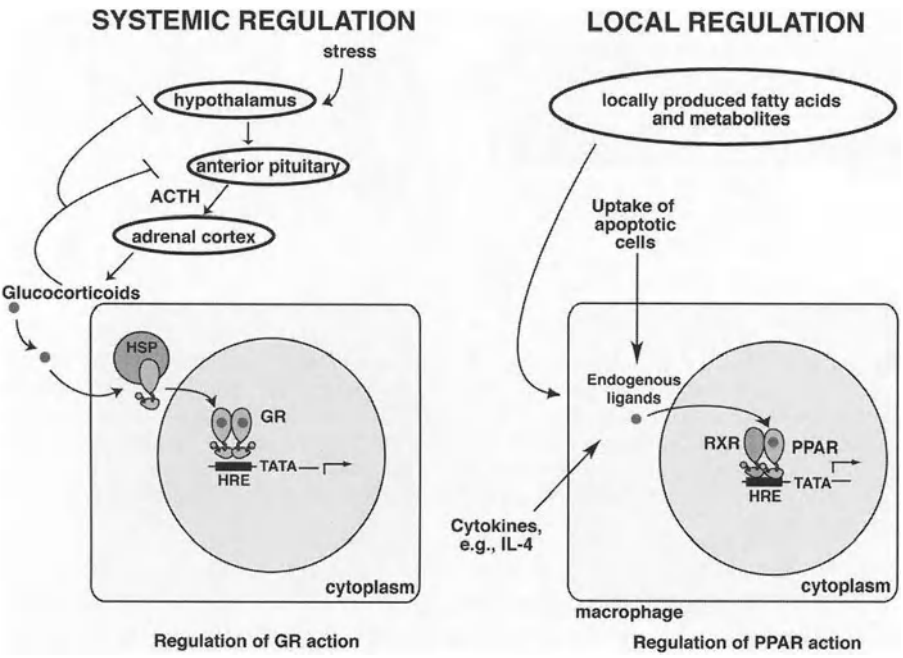

Fig. 2 Systemic and local regulation of macrophage gene expression by nuclear receptors. Classical steroid hormone receptors, such as the glucocorticoid receptor, regulate gene expression in response to circulating hormones that are produced under the control of the hypothalamic/pituitary axis. Adopted orphan receptors, such as PPARs and LXRs (not shown), regulate macrophage gene expression in response to intracellular metabolites of fatty acids and cholesterol, respectively. The production of these ligands may be influenced by local cytokines and other classes of signaling molecules

is essential for coactivator recruitment, ligands that fit in the ligand-binding pocket, but distort the AF2 position, offer the potential to act as antagonists or as selective modulators of nuclear receptor activity (Brzozowski et al. 1997; Shiau et al. 1998). While this principle has been clearly demonstrated for the anti-estrogens raloxifene and raloxifene, it remains to be generalized to the entire nuclear receptor family.

1.2 Functional Classification

The spectrum of nuclear receptors can be subdivided into three sub-families based on their ligand-binding properties and physiological roles (Chawla et al. 2001). The classical steroid/thyroid hormone receptors, exemplified by glucocorticoid and estrogen receptors, define the first and most extensively characterized subfamily (Evans 1988). The so-called orphan receptors define a subfamily at the opposite end of the nuclear receptor spectrum in that they exhibit conserved features of the nuclear receptor superfamily, but have not yet been

linked to naturally occurring ligands. A subset of orphan nuclear receptors, such as RORα and Nurr77, have been suggested to play important roles in regulating lymphocyte function and survival (Winoto and Littman 2002), but roles in macrophages remain relatively unexplored. The third sub-family consists of "adopted" orphan receptors. These receptors were initially identified as orphan receptors, with subsequent studies leading to the identification of naturally occurring ligands and physiological roles. Members of this subfamily that have been recently linked to regulation of macrophage gene expression include the PPARs and LXRs (Chawla et al. 2001).

The transcriptional activities of steroid hormone and adopted orphan receptors are regulated by small lipophilic molecules. In the case of the steroid hormone receptors, the regulatory ligands are classic endocrine hormones, produced in glandular tissues in response to systemic physiologic circuits [e.g., the hypothalamic-pituitary-adrenal axis Fig. 2)]. Steroid hormones diffuse into cells and bind to their cognate receptors in target tissues with sub-nanomolar affinities. In contrast, the adopted orphan receptors tend to be activated by metabolites of cholesterol and fatty acids that are produced within the cell and bind with relatively low affinity. Thus, adopted orphan receptors appear to function as effectors of autocrine or paracrine signaling events (Fig. 2). For both steroid hormone and adopted orphan receptors, numerous high-affinity synthetic ligands have been developed, some of which are in widespread clinical use. For example, the synthetic GR agonists dexamethasone and prednisone are extensively utilized as potent anti-inflammatory agents, and the synthetic PPARγ agonist rosiglitazone is used for treatment of type 2 diabetes mellitus.

1.3 Transcriptional Activities of Nuclear Receptors

Nuclear receptors have been shown to regulate transcription by three general mechanisms. The prototypic activity of nuclear receptors is to activate transcription in a ligand-dependent manner following direct binding to DNA response elements in promoter or enhancer regions of target genes (Figs. 2 and 3, *ligand-dependent transactivation*). Ligand-dependent transactivation has been linked to the recruitment of coactivator complexes that modify chromatin structure and facilitate assembly of general transcriptional machinery at the promoter (Glass and Rosenfeld 2000; McKenna and O'Malley 2002). A large number of coactivator complexes have been identified, and it is hypothesized that combinatorial usage of these complexes provides the basis for cell type-specific, gene-specific, and signal-specific transcriptional responses. Nuclear receptors can negatively regulate gene expression by inhibiting the activities of other classes of signal-dependent transcription factors, such as members of the NF-κB and AP-1 families (Jonat et al. 1990; Yang-Yen et al. 1990; Schule et al. 1991; Ray and Prefontaine 1994; Helmberg et al. 1995) (Fig. 3, *ligand-dependent transrepression*). Several mechanisms have been suggested to account for this activity, but unifying principles remain to be elucidated. Third, several of the adopted or-

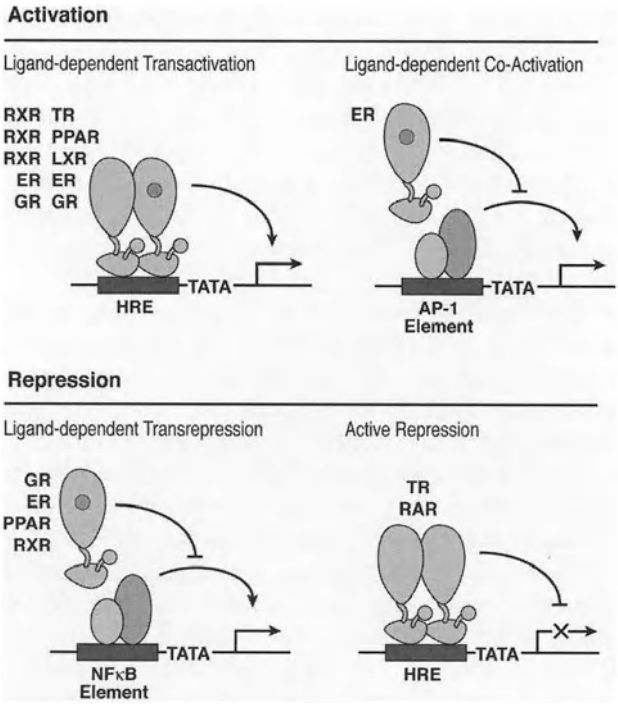

Fig. 3 Transcriptional activities of nuclear receptors. *Ligand-dependent transactivation* is mediated by the binding of nuclear receptor dimers or heterodimers to hormone response elements in target genes. Nuclear receptors can directly repress transcription in the absence of ligand (*ligand-independent repression*) or indirectly inhibit transcription in a ligand-dependent manner by inhibiting the activities of other transcription factors such as nuclear factor (NF)-κB (*ligand-dependent transrepression*). Other mechanisms of action may also exist. For example, the estrogen receptor has been demonstrated to activate transcription from activator protein (AP)-1 elements by serving as a coactivator (*ligand-dependent coactivation*)

phan receptors, exemplified by retinoid acid receptor (RAR) and thyroid receptor (TR), actively repress transcription of direct target genes in the absence of ligands (Fig. 3, *ligand-independent repression*) (Damm et al. 1989; Sap et al. 1989; Datta et al. 1992). This activity has been linked to the recruitment of corepressor complexes that function to antagonize the actions of coactivator complexes (Chen and Evans 1995; Horlein et al. 1995). Not all nuclear receptors exhibit this entire spectrum of transcriptional activities and the possible transcriptional effects of a single nuclear receptor vary in a cell-specific manner. In addition to these general mechanisms of nuclear receptor action, specific nuclear receptors have been demonstrated to regulate transcription by other mechanisms. For example, the estrogen receptor can in some contexts interact with AP-1 proteins and function as a coactivator (Fig. 3, *ligand-dependent coactivation*) (Paech et al. 1997).

2
Nuclear Receptor Functions in Macrophages

Studies of nuclear receptor function in the macrophage suggest three general physiologic roles. One role is the negative regulation of inflammatory responses mediated by AP-1 and NF-κB family members. Emerging evidence suggests that these actions represent important functions of GRα, ERα, and PPARs in the macrophage. A second major role that has emerged involves regulation of lipid homeostasis by LXRs and PPARs. Third, a smaller subset of nuclear receptors, exemplified by the vitamin D receptor, have been found to influence specialized programs of macrophage differentiation, such as osteoclast formation.

While GRα, VDR, RXRα, and LXRβ appear to be constitutively expressed in macrophages, ERα and PPARγ have both been shown to be increased during macrophage differentiation (Ricote et al. 1997; Cutolo et al. 2001). In addition, PPARγ is upregulated by multiple stimuli in the macrophage, including oxidized low-density lipoprotein (LDL), granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and interleukin (IL)-4 (Nagy et al. 1998; Ricote et al. 1998; Huang et al. 1999). LXRα expression is positively regulated by its oxysterol ligands, providing a positive feedback mechanism for maintenance of cholesterol homeostasis (Kohro et al. 2000; Laffitte et al. 2001).

2.1
Systemic and Endogenous Ligands

Ligand availability represents one of the most important determinants of nuclear receptor activity. Thus far, the macrophage appears to possess mechanisms for the autocrine or paracrine production of three distinct classes of ligands. First, although estrogen is largely an endocrine hormone regulated by the hypothalamic-gonadal axis, differentiated macrophages express and regulate the enzyme aromatase, capable of converting serum dehydroepiandrosterone (DHEA) into the immunomodulatory steroids estrogen, 3β,17β-androstenediol, and androstenedione (Schmidt et al. 2000). Second, PPARγ can be activated by a variety of fatty acid metabolites and oxidation products. These include the linoleic and arachidonic acid metabolites 13-HODE, 15-HETE and 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (Forman et al. 1995; Kliewer et al. 1995; Forman et al. 1997; Kliewer et al. 1997; Nagy et al. 1998). Both 13-HODE and 15-HETE have been identified as components of oxidized LDL, which has itself been shown to upregulate PPARγ expression and induce its transcriptional activity (Nagy et al. 1998; Ricote et al. 1998). In addition, 13-HODE and 15-HETE can be enzymatically generated by the IL-4 inducible 12/15-lipoxygenase, suggesting that IL-4 can coordinately regulate both the expression and activity of PPARγ (Huang et al. 1999). Third, LXRs are activated by cholesterol metabolites (Peet et al. 1998). In the macrophage, the enzyme 27-hydroxylase is able to modify cholesterol into a ligand for both LXRα and LXRβ (Babiker et al. 1997; Fu et al. 2001). Therefore,

nuclear receptors provide mechanisms for regulating macrophage gene expression in response to changes in cellular lipid homeostasis and the production of arachidonic acid metabolites that occur during the evolution of inflammatory responses.

2.2 Anti-inflammatory Effects of GRα, ERα, PPARγ, and VDR

The striking ability of GRα agonists to inhibit inflammatory responses is one of the best-documented effects of nuclear receptor ligands on macrophage physiology. Endogenous glucocorticoids are released in response to a variety of stressors (starvation, pain, trauma, infection, etc.) and are essential for maintenance of homeostatic functions (Fig. 2) (Schimmer and Parker 1996). Although in widespread clinical use for the treatment of a variety of inflammatory diseases including rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, psoriasis, eczema, asthma and transplant rejection, the mechanistic basis of the anti-inflammatory actions of glucocorticoids remains poorly understood (Barnes 1995; McKay and Cidlowski 1999). Accumulating evidence suggests that these effects largely result from inhibition of signal-dependent transcription factors that mediate inflammatory programs of gene activation, particularly NF-κB and AP-1 family members (Herrlich and Ponta 1994; McKay and Cidlowski 1999). NF-κB is a transcription factor that is activated in multiple cell types by inflammatory stimuli such as bacterial lipopolysaccharides (LPS) or interleukins such as IL-1β (Barnes and Karin 1997). Like GRα, classical NF-κB activation involves the removal of a cytoplasmic inhibitor (IκB) and the nuclear translocation of NF-κB dimers or heterodimers to cognate κB elements in the promoter or enhancer regions of target genes. AP-1 transcriptional activity can also be activated by a number of inflammatory stimuli as well as by phorbol esters [e.g., 12-*O*-tetradecanoylphorbol-13-acetate (TPA)]. These general mechanisms allow for the regulated expression of a wide variety of growth factors (GM-CSF), cytokines (IL-1, IL-6, IL-8, TNF-α, MCP-1), and inflammatory mediators [inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)2] in macrophages and other cell types (McKay and Cidlowski 1999). Many of these inflammatory genes can be repressed by GRα activity. Genes that are strongly repressed by GRα agonists include GM-CSF (Adcock and Barnes 1996), TNF-α, (Joyce et al. 1997), IL-1, IL-6, IL-8, IL-12 (Almawi et al. 1996), iNOS (Kleinert et al. 1996; Tanaka and Fujita 1997) and COX2 (Koehler et al. 1990). Despite the requirement for both the GRα, LBD, and DBD for transrepression activity, the majority of the promoters and enhancers for these genes do not contain functional glucocorticoid response elements (GREs) (Caldenhoven et al. 1995; Scheinman et al. 1995; McKay and Cidlowski 1999). These observations suggest that GRα-mediated transrepression of NF-κB and AP-1 involves mechanisms that are distinct from the classical GRα transactivation of target genes (Heck et al. 1997) (Fig. 3). Interestingly, activation of the AP-1 and NF-κB pathways results in reciprocal antagonism of the classical GRα transactivation of HRE-con-

taining promoters (Jonat et al. 1990; Caldenhoven et al. 1995). This has raised the possibility that NF-κB and GRα compete for a limiting supply of co-activator complexes and use similar mechanisms for mutual repression (Kamei et al. 1996; Sheppard et al. 1998; McKay and Cidlowski 2000). Modification of the degree of phosphorylation of the C-terminal repeat of RNA polymerase II at NFκB target genes has also been suggested as the basis for GR-mediated transrepression (Nissen and Yamamoto 2000). Regardless of the mechanistic details, these opposing transcription factors appear to mutually regulate the other's function, maintaining a balance of inflammatory and anti-inflammatory responses in the macrophage and other cells that regulate immune responses, including endothelial cells and lymphocytes.

Like GRα, ERα and PPARγ have also been shown capable of antagonizing the expression of an overlapping set of NF-κB and AP-1 regulated genes in macrophages (Frazier-Jessen and Kovacs 1995; Stein and Yang 1995; Jiang et al. 1998; Ricote et al. 1998). This transrepression function has also been shown to require elements of both the LBD and DBD, but not direct binding to HREs in the enhancer or promoter regions of these genes (Li et al. 2000; Valentine et al. 2000). VDR also inhibits inflammatory gene expression, but has been suggested to exert these effects by inducing the expression of both transforming growth factor (TGF)-β and IL-4 (Deluca and Cantorna 2001). These factors modulate transcriptional programs that evolve during an immune response and act to antagonize the effects of many classical inflammatory signals.

The findings that nuclear receptors possess the ability to modulate inflammatory immune responses have raised the possibility that agonists for ERα, PPARγ, or VDR might have clinical applications as anti-inflammatory drugs. Currently, PPARγ ligands have been shown to ameliorate inflammation in animal models of inflammatory bowel disease (Su et al. 1999; Desreumaux et al. 2001), atherosclerosis (Li et al. 2000; Chen et al. 2001; Claudel et al. 2001; Collins et al. 2001), experimental autoimmune encephalomyelitis (Diab et al. 2002), arthritis (Setoguchi et al. 2001), and psoriasis (Ellis et al. 2000). Likewise, clinical evidence has long been mounting that estrogens play a key role in modulating atherogenesis independent of their effects on lipid metabolism (Reckless et al. 1997; Cushman et al. 2001). In addition, 1,25-dihydroxyvitamin D_3 has been shown to ameliorate experimental autoimmune encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease (Deluca and Cantorna 2001).

2.3 Regulation of Cholesterol Homeostasis by LXRs

Tight regulation of cellular cholesterol levels is essential for the maintenance of a diverse range of normal cellular functions. In most cells, cholesterol availability is determined by the sum of de novo biosynthesis and uptake from lipoproteins via the LDL receptor (Brown and Goldstein 1986). These processes are regulated by a negative feedback system involving the sterol response element

binding proteins (SREBPs), which are transcription factors that stimulate expression of the LDL receptor gene as well as genes involved in cholesterol and fatty acid biosynthesis (Brown and Goldstein 1997). When cellular cholesterol levels become elevated, SREBPs are inactive, leading to decreased cholesterol availability. In addition, elevated cholesterol levels have recently been demonstrated to stimulate cholesterol efflux by inducing the expression of sterol transporters such as ABCA1. Stimulation of efflux pathways by elevated cholesterol levels has been linked to the adopted orphan receptors, LXRα and LXRβ. These receptors bind with RXRs to response elements in target genes such as ABCA1 and are activated by oxysterols that are thought to accumulate in hypercholesterolemic cells (Peet et al. 1998; Chawla et al. 2001). The LXR–ATP-binding cassette (ABC) pathway appears to be particularly important in the macrophage. Mutations in the ABCA1 gene result in Tangier disease, which is characterized by lipid filled macrophages in tissues such as the tonsils, and extremely low levels of circulating high-density lipoproteins (HDL) (Young and Fielding 1999; Tall and Wang 2000). Because one essential function of the macrophage is the phagocytosis and degradation of cholesterol-containing apoptotic and necrotic cells, the macrophage may utilize the LXR-ABC pathway as a critical feed-forward mechanism for disposing of such cellular by-products. This system appears to be overwhelmed or inactivated in foam cells of atherosclerotic lesions, which contain massive amounts of cholesterol derived from modified lipoproteins that are taken up by scavenger receptors. LXR alpha expression is induced in macrophages by PPARγ ligands, which may account for some to the antiatherogenic effects observed in murine models (Chawla et al. 2001).

2.4 Effects of Nuclear Receptors on Macrophage Differentiation and Specialized Functions

One of the most intriguing characteristics of the macrophage lineage is its ability to give rise to a family of related cells that execute specialized roles, such as Kupffer cells, osteoclasts, and microglial cells (Gordon 1995). For example, recent work has found that mature bone resorbing osteoclasts can be induced to differentiate from cells of the monocyte lineage when stimulated with osteoblast products M-CSF and receptor activator of NF-κB ligand (RANKL) (Roodman 1999). This differentiation program can be inhibited by the addition of either an estrogenic ligand (Shevde et al. 2000) or a PPARγ ligand (Bendixen et al. 2001). In contrast, glucocorticoids also decrease bone resorption, but appear to do so by increasing osteoclast apoptosis (Dempster et al. 1997). While mechanisms for these effects are not yet fully elucidated, current models have focused on the inhibition of NF-κB activity, an essential stimulus for osteoclastogenesis.

Like osteoclasts, dendritic cells can be induced to differentiate from macrophages in vitro, but in the presence of different stimuli: GM-CSF and IL-4. This differentiation program can be largely prevented by either the presence of corticosteroids, anti-estrogens (Komi and Lassila 2000) or vitamin D analogs (Griffin

et al. 2000; Piemonti et al. 2000). Vitamin D analogs have also been shown to enhance monocyte-to-macrophage differentiation, suggesting that they play a role in determining a balance in the monocyte lineage developmental choices (Nakajima et al. 1996). The physiological significance of these findings remains to be established in vivo.

Recent genetic screens have also identified mutations in the VDR as susceptibility markers for infection with the intracellular pathogens *Mycobacterium tuberculosis* and *M. leprae* (Roy et al. 1999; Bellamy 2000). This relationship could result from VDR biasing a T helper (Th)2 immune response or from as yet uncharacterized mechanisms. However, it does suggest that normal VDR contributions to macrophage function are an important part of regulatory mechanisms necessary to deal with these and perhaps other pathogens.

3 Conclusions and Future Directions

Several nuclear receptors are expressed in macrophages and ligands for these receptors have been documented to influence inflammatory responses, specialized macrophage functions, and lipid homeostasis. While these findings have important biological and pharmacological implications, regulation of macrophage gene expression by members of the extended nuclear receptor family remains relatively unexplored. Even for the best-characterized nuclear receptors, emerging information on the ability of synthetic ligands to alter the specificity of coactivator and corepressor recruitment raises new possibilities for the development of novel pharmaceutical agents. Advances in the understanding of mechanisms responsible for transcriptional activation and repression by nuclear receptors may allow the development of selective nuclear receptor modulators that regulate a defined subset of target genes. For example, it may be possible to develop ligands for the glucocorticoid receptor that retain the ability to inhibit NF-κB, but do not have gluconeogenic activities (Vayssière et al. 1997). Such ligands would be likely to exert anti-inflammatory effects without many of the limiting side effects of currently available steroid hormone analogs. The ability to selectively modulate nuclear receptor function may prove to be of therapeutic benefit in a wide range of human diseases in which macrophages play important roles, including atherosclerosis, osteoporosis, and chronic inflammatory diseases.

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Relationships Between Reactive Oxygen Species and Reactive Nitrogen Oxide Species Produced by Macrophages

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Abstract The armature of macrophages includes systems that catalytically produce a variety of chemical agents that help form the core of the innate inflammatory response. Compounds derived from oxygen are collectively known as reactive oxygen species (ROS), while those generated from nitrogen and oxygen are termed reactive nitrogen oxide species (RNOS). Both ROS and RNOS participate in cytotoxic mechanisms designed to kill pathogens. These systems require tight regulatory control to deter vascular abnormalities and host cell damage. ROS and RNOS also function to modulate a broad array of signaling pathways that shape adaptive immune responses. Effective pharmacological intervention of bystander injury elicited by ROS and RNOS will require an understanding of the specific interrelationships between these agents and how they factor into the various phases of inflammatory responses, which may be unique to different leukocyte subpopulations within each organ system.

Keywords Hydrogen peroxide, Myeloperoxidase, NADPH oxidase, Nitric oxide, Nitric oxide synthase, Nitrogen dioxide, Nitrosation, Nitration, Nitrite, Oxidation, Peroxynitrite, Superoxide

1 Introduction

The reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) produced by macrophages play key roles in innate immune responses and the development of specific adaptive immunity. This review will focus on the major enzymatic systems in macrophages that generate ROS and RNOS. The factors that influence the secondary interactions of ROS and RNOS following their formation will be emphasized. An excellent overview of ROS and RNOS interactions has been previously presented in the *Handbook of Experimental Pharmacology* series (Wink et al. 2000).

2 Reactive Oxygen Species

2.1 Superoxide and NADPH Oxidase

The major source of ROS produced by activated leukocytes is the multicomponent enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyzes the production of superoxide (O_2^-) by the one-electron reduction of oxygen (Sbarra and Karnovsky 1959; Babior 1973, 1999; Root and Cohen 1981; Tauber et al. 1983; Garcia and Segal 1988; Sies and de Groot 1992; Bastian and Hibbs 1994; Robinson and Badwey 1994, 1995; Rosen et al. 1995; Clark 1999). Although the preponderance of data are derived from neutrophils, the pivotal importance of NADPH oxidase in combating pathogens has been amply demonstrated in macrophages. In contrast to vanquishing pathogens, O_2^- production from NAD(P)H oxidase in non-phagocytes participates in signal transduction and the regulation of blood pressure as well as atherosclerotic proliferation. Activation of NADPH oxidase in lymphocytes may largely serve a mitogenic role (Lee et al. 1998; Devadas et al. 2002). Endothelium and vascular smooth muscle cells in kidney (Wilcox 2001), heart (Griendling et al. 2000), and lung (Brar et al. 2002) also possess a relatively lower level of NAD(P)H oxidase activity that is somewhat homologous to the enzyme assembly in leukocytes.

Phagocytes increase oxygen consumption in response to a variety of stimuli; however, the term respiratory burst is a misnomer in that oxygen usage is not strictly related to mitochondrial respiration. Rather, glucose is consumed via the pentose phosphate pathway forming NADPH, which donates electrons for the oxidase reaction. For each NADPH, two electrons are shuttled through the flavin portion of the complex for the univalent reduction of oxygen (Eq. 1).

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^- \quad (1)$$

A commonly used inhibitor of the electron transfer reaction is diphenylene iodonium (Cross and Jones 1986); however, this reagent also has inhibitory interaction with mitochondrial complex I (Ragan and Bloxham 1977).

A multitude of stimuli can prompt assembly and activation of the NADPH oxidase complex, which in phagocytes comprises five phox (phagocyte oxidase) components. An initial step involves phosphorylation of cytosolic $p47^{phox}$, which in association with $p40^{phox}$ and $p67^{phox}$ is mobilized to the plasma membrane. These subunits subsequently associate with the $p22^{phox}$and $p91^{phox}$ dimer, which comprise the cytochrome b_{558}. Alternatively, subunits may segment via intracytoplasmic vesicles directly to phagolysosomes. Activity of the NADPH oxidase complex is further modified by interaction with small G proteins Rac (Rho family, cytosolic) and Rap (Ras family, membrane). Therefore, NADPH oxidase activity can be regulated through a multitude of kinase and G protein signaling cascades (Bromberg et al. 1991; Babior 1999; Prada-Delgado et al. 2001). Stimulation of neutrophils with phorbol ester resulted in a rapid onset of O_2^- formation with a rate of 0.78 nmol/min/10^6 cells, while zymosan exposure elicited a slower onset and 10-fold lower rate of production (Roubaud et al. 1998). Cytokines, such as interferon (IFN)-γ or tumor necrosis factor (TNF)-α, can prime expression of signal transduction components in phagocytes resulting in augmented NADPH oxidase activity upon a secondary stimulus (Robinson and Badwey 1994). In addition to soluble factors, cues from the surrounding matrix in conjunction with changes in cell morphology influence trafficking, activation, and catalytic efficiency of NADPH oxidase (Berton and Gordon 1983; Wymann et al. 1989; Nathan et al. 1989; Nauseef et al. 1991; Zhou and Brown 1993; Berton et al. 1996; Hampton et al. 2002).

2.2 Hydrogen Peroxide

2.2.1 Haber-Weiss Cycle

In general, activated NADPH oxidase complex vectorially generates O_2^- on the extracellular face of the plasma membrane. Within minutes of pathogen internalization, fusion events occur to join the NADPH oxidase complex with the phagolysosome (DeLeo et al. 1999). Alternatively, subunits may segment via intracytoplasmic vesicles directly to phagolysosomes. Within this compartment, conditions are favorable for the dismutation of O_2^- to form hydrogen peroxide (H_2O_2; Eq. 2).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

Much of the cytotoxicity associated with NADPH oxidase activity is due to secondary generation of H_2O_2 in conjunction with other components of the phago-

lysosome (Klebanoff 1967; Nanda et al. 1994; Pacelli et al. 1995; Reeves et al. 2002). Microbes have evolved specific defenses against ROS (Prada-Delgado et al. 2001; Vazquez-Torres and Fang 2001).

Dismutation of O_2^- to H_2O_2 is catalytically accelerated to near diffusion control by the superoxide dismutase (SOD) family of isoenzymes (Fridovich 1995, 1999). Cytosolic and mitochondrial SOD in conjunction with peroxisomal catalase, which catalytically forms water from H_2O_2, categorically serves a protective role. Extracellular Cu/Zn SOD (tetrameric glycoprotein isoforms called type A, B, and C) is also present in the tissue matrix and bound to heparin sulfate proteoglycans on the surface of various cell types (Halliwell and Gutteridge 1990; Abrahamsson et al. 1992). Therefore, under inflammatory conditions, generation of O_2^- by NADPH oxidase may lead to formation of H_2O_2 at these intercellular sites, which may influence the redox environment and shape the inflammatory response. Low-dose exposure of macrophages to exogenous H_2O_2 has been shown to elicit specific functional changes (Gamaley et al. 1994, Rhee 1999; Forman and Torres 2001a,b).

In contrast to O_2^- (pK_a=4.3; Fridovich 1995), H_2O_2 may be considered the traveling ROS in that it is freely permeates through cellular membranes. Generation of H_2O_2 through O_2^- formation by NADPH oxidase can cause tissue injury when free-transition metal (M^n; such as Fe^{3+}, Cu^{2+}) catalysts are present (Eqs. 3, 4; Halliwell and Gutteridge 1984, 1999; Hibbs et al. 1984).

$$M^n + O_2^- \rightarrow M^{n-1} + O_2 \tag{3}$$

$$M^{n-1} + H_2O_2 \rightarrow M^n + {}^{\cdot}OH + OH^- + O_2 \text{ (Fenton reaction)} \tag{4}$$

This reaction pathway has become known as the Haber-Weiss cycle (Haber and Weiss 1934), which links O_2^- generation of reduced transition metals (M^{n-1}) to putative formation of the highly reactive hypervalent metal species or hydroxyl radical ($^{\cdot}OH$) via H_2O_2 (Fenton 1894). However, the existence of $^{\cdot}OH$ versus production of alternate oxidizing intermediates remains an area of debate (Koppenol 1985; Wink et al. 1994). For $^{\cdot}OH$ to be an effective bactericide, this ROS would likely need to be generated in the immediate vicinity of the bacteria within the phagosome (Lymar and Hurst 1995). These data emphasize the differences in relative toxicities of ROS and RNOS when determined in the context of cellular and extracellular compartments. In addition to the NADPH oxidase system, lipoxygenase-catalyzed reactions may account for a significant portion of ROS formation by activated macrophages (e.g., 30%; Hume et al. 1983).

2.2.2 Myeloperoxidase

Myeloperoxidase (MPO) is a tetrameric glycosylated heme protoporphyrin-containing enzyme that is stored primary in azurophilic granules (Nauseef and Malech 1986; Heinecke et al. 1993; Kettle and Winterbourn 1997; Podrez et al.

2000). Upon stimulation, MPO can be released into the intercellular space or transport into the phagolysomal pathway and works in tandem with NAD(P)H oxidase pathways. Hydrogen peroxide is required to convert the resting-state, ferric heme of MPO by two electron equivalents to the hypervalent ferryl π cation radical termed compound I. In the presence of halides such as Cl^-, Br^-, and I^-, and the pseudohalide SCN^-, compound I is reduced in a single two-electron step to regenerate resting-state (MPO-Fe^{3+}) and form the corresponding hypohalous acid (HOX). Alternatively, MPO can oxidize nitrite (NO_{2-}) by one electron to give the RNOS nitrogen dioxide (NO_2) and the MPO intermediate compound II. Subsequently, an additional nitrite molecule will reduce compound II by one electron to regenerate the resting state and produce a second NO_2 (Eiserich et al. 1996; Abu-Soud and Hazen 2000; van Dalen et al. 2000). MPO-catalyzed formation of NO_2 can lead to nitration of aromatic compounds such as tyrosine (see sections entitled "NO and O_2^-" and "NO_2^- and H_2O_2"). Numerous other unknown substrates for peroxidation by MPO likely exist. Similar to NADPH oxidase, the presence of transition metals can enhance MPO-catalyzed oxidant production (Ramos et al. 1992).

The roles MPO plays in macrophage biology remain an area of debate. MPO activity in monocytes declines rapidly upon adherence and differentiation in vitro (Nakagawara et al. 1981). However, development of peroxidatic activity in the rough endoplasmic reticulum (ER) and perinuclear cisternae has been observed 2 h after monocyte adherence to serum- or fibrin-coated surfaces (Bodel et al. 1977). MPO immunoreactivity is associated with activated macrophages within human atherosclerotic vascular tissue (Daugherty et al. 1994). Subsequent studies have shown that macrophage MPO mediates modification of LDL cholesterol causing aberrant lipoprotein oxidation, aggregation, and uptake (Hazen et al. 1996, 1999; Chisolm et al. 1999; Hazen 2000; Podrez et al. 2000).

In addition to adherence, MPO activity in monocytes/macrophages is strongly influenced by numerous soluble immune factors. CD14-positive monocytes purified from peripheral blood monocytes developed into dendritic cells with potent antigen-presenting capacity following exposure to cytokines granulocyte-monocyte colony-stimulating factor and interleukin (IL)-4, but also retained significant amounts of MPO (Pickl et al. 1996), while a downregulation of expression has been observed in other preparations (Tsuruta et al. 1996). Likewise, macrophage exposure to exogenous MPO results in alterations in function, such as TNF-α production (Shepherd and Hoidal 1990; Lefkowitz et al. 1992; Lefkowitz and Lefkowitz 2001). Macrophages play an important role in the clearance of neutrophils (Savill et al. 1989) and remove neutrophil-derived MPO via mannose receptor-mediated uptake (Biggar and Sturgess 1976; Shepherd and Hoidal 1990). MPO of both endogenous (Rodrigues et al. 2002) and exogenous origin (Leung and Goren 1989; Lincoln et al. 1995) may incorporate into the macrophage phagosome and augment bacterial killing. A mixture of enzymatically active and effete forms of MPO released during neutrophil degranulation may coordinate a variety of macrophage functions (Bradley et al. 1982; Lefkowitz et al. 2000; Lefkowitz and Lefkowitz 2001).

These data raise an important point for consideration. The role of ROS and RNOS (see "Reactive Nitrogen Oxide Species" below) during an immune response is highly dependent on the tissue and the phase of the response. Activation of NADPH oxidase (either in the absence or presence of SOD) results in formation of H_2O_2, which in turn, provides electrons for MPO-catalyzed oxidation of substrates. However, each of these components does not necessarily have to present within the macrophage. Macrophage interaction with surrounding leukocytes, parenchymal cells, and their products profoundly shapes the pattern of ROS and RNOS that are subsequently formed, thereby impacting the immune response on multiple levels (Klebanoff 1980).

3
Reactive Nitrogen Oxide Species

3.1
Nitric Oxide and Nitric Oxide Synthase

RNOS originate with nitric oxide (NO) biosynthesis catalyzed by the nitric oxide synthase (NOS) family of isozymes, an exception being a potential contribution from dietary nitrite (NO_2^-) and nitrate (NO_3^-). The discovery of NO in biology and pathophysiology was borne out of metabolic studies with macrophages. In 1985, Stuehr and Marletta showed that activation of macrophages with bacterial lipopolysaccharide (LPS) caused a marked increase in NO_{2-} and NO_{3-} formation (Stuehr and Marletta 1985). Hibbs and colleagues subsequently observed that the cytostatic and tumoricidal capacity of macrophages stimulated with cytokines in vitro was dependent on the presence of L-arginine in the culture medium (Hibbs et al. 1987b). Moreover, this capacity was linked to the conversion of L-arginine to L-citrulline with formation of NO_2^- rather than the known urea cycle pathway involving loss of the guanidino-carbon of L-arginine to urea via arginase and subsequent conversion of L-ornithine to L-citrulline by ornithine transcarbamoylase (Hibbs et al. 1987a). These studies paved the way for the identification of NO as the effector molecule (Hibbs et al. 1988; Marletta et al. 1988) and cloning of the inducible isoform of nitric oxide synthase (iNOS, often termed NOS II) from macrophages (Lowenstein et al. 1992; Lyons et al. 1992; Xie et al. 1992). The biosynthesis and function of NO in the immune system has been extensively reviewed in the *Handbook of Experimental Pharmacology* series (Bogdan 2000; Zamora and Billiar 2000). Several excellent sources on the structure and function of NOS are available (Geller and Billiar 1998; Weinberg 1998; Stuehr 1999; Stuehr and Ghosh 2000; Alderton et al. 2001).

The human iNOS gene contains 26 exons and encodes a 131-kDa protein (Geller et al. 1993; Chartrain et al. 1994). Interestingly, an alternative splice variant of iNOS has been detected in human lung epithelia (Eissa et al. 1998). NOS can be divided into three functional domains: (1) an amino-terminal oxygenase domain that binds heme, L-arginine, and tetrahydrobiopterin (BH_4); (2) a calmodulin (CaM)-binding domain; and (3) a carboxy-terminal reductase domain

that binds flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH, which facilitates electron transfer to the oxygenase domain. The catalytic cycle of NOS proceeds in two separate successive monooxygenase reactions with the overall stoichiometry given in Eqs. 5 and 6).

$$\mathrm{L-arginine} + \mathrm{NADPH} + \mathrm{H^+} + \mathrm{O_2} \rightarrow \mathrm{N^w{-}hydroxy-L-arginine} + \mathrm{NADP^+} + \mathrm{H_2O} \quad (5)$$

$$N^w\mathrm{-hydroxy-L-arginine} + 0.5\mathrm{NADPH} + 0.5\mathrm{H^+} + \mathrm{O_2} \rightarrow \mathrm{L-citrulline} + 0.5\mathrm{NADP^+} + \mathrm{H_2O} + \mathrm{NO} \quad (6)$$

To balance these equations for NO formation, NADPH must contribute 1.5 reducing equivalents. Several groups have proposed that the true stoichiometry is one NADPH consumed per monooxygenase cycle resulting in HNO (nitroxyl) as the product (Hobbs et al. 1994; Schmidt et al. 1996; Adak 2000). HNO has a reactivity pattern uniquely different from NO (Hughes 1999; Bartberger et al. 2001; Miranda et al. 2001, 2002; Espey et al. 2002a). Consequently, catalytic production of HNO by NOS in lieu of NO would influence a different array of cellular targets (Ma et al. 1999; Colton et al. 2001; Paolocci et al. 2001; Espey et al. 2002a).

All NOS isoforms require CaM binding for enzymatic activity. However, iNOS is distinguished from the neuronal and endothelial NOS isoforms (NOS-I, NOS-III) in that CaM is tightly bound at basal calcium levels and therefore does not require higher calcium transients for activation. In addition, iNOS lacks sequences present in the FMN-binding subdomain of constitutive NOS isoforms that may serve to destabilize electron transfer to the heme pocket at lower basal calcium levels (Salerno et al. 1997). Indeed, only an approximately 50% homology exits between iNOS and the neuronal and endothelial NOS isoforms (Ganster and Geller 2000; Zamora et al. 2000). Further distinction of iNOS resides in formation of the homodimer, a requisite step for l-arginine catalysis. In addition to dimer stabilization by heme and l-arginine, iNOS may be particularly sensitive to BH_4 binding, which facilitates alignment of the subunits in a head-to-head manner (Venema et al. 1997; Stuehr 1999; Chen et al. 2002).

While expression and activity of iNOS in rodents can be readily achieved in the laboratory, demonstration has been relatively more difficult in human leukocyte preparations. However, numerous studies have clearly shown iNOS plays a pivotal role in human macrophages (MacMicking et al. 1997). In septic patients, iNOS activity was markedly increased in putrescent areas of the systemic vasculature containing macrophages and expression of TNF-α and IL-1β (Annane et al. 2000). Human macrophages treated ex vivo with LPS and IFN-γ yielded an iNOS-specific amplification product by reverse transcriptase polymerase chain reaction (RT-PCR) (Reiling et al. 1994). IFN-γ, α or IL-4 augmented polyribonucleotide-induced NO_2^- production by human monocyte-derived macrophages (Snell et al. 1997). Alveolar macrophages isolated from lavage fluid

of patients with tuberculosis have been shown to produce NO (Steiner et al. 1996). IFN-α treatment of macrophages or administration of IFN-α to hepatitis C patients in vivo increased expression of iNOS mRNA, protein, and activity concomitant with NO production (Sharara et al. 1997; Weinberg 1998). Apolipoprotein-E augmented NO production from activated human microglia, while amyloid-beta peptide blocked this effect (Vitek et al. 1997). Many cells in addition to macrophages are capable of iNOS expression including astrocytes, chondrocytes, hepatocytes, neurons, neutrophils, skeletal muscle, vascular smooth muscle, vascular endothelial cells, and several cancers.

In general, macrophages initiate synthesis of iNOS after immune stimulation (Nathan and Xie 1994; MacMicking et al. 1997; Bogdan 2000; Clancy et al. 1998; Ganster and Geller 2000; Taylor and Geller 2000; Zamora et al. 2000). However, constitutively expressed iNOS has been observed in some cell types (Mannick et al. 1994; Guo et al. 1995) including macrophages (Amin et al. 1995). The signal transduction pathways mediated by IFN are of particular importance. The Jak-STAT cascade is initiated by IFN binding to cell surface receptors with intrinsic or receptor-associated tyrosine kinase activity (Jak), which mediates phosphorylation of cytosolic proteins collectively known as STATs. STAT members activated in this manner form homo- and heterodimeric complexes that translocate to the nucleus, interact with other DNA-binding proteins (e.g., interferon regulatory factors) and bind specific sequences in the iNOS promoter region (e.g., gamma activation sequences, interferon response sequence elements). Exposure to IFN primes macrophages for expression of iNOS upon secondary activation (Espey et al. 2000b). Additional immune modulators such as TNF-α, IL-1β and pathogen products (e.g., LPS) are potent secondary stimulants. These agents predominantly regulate transcription of iNOS through the NF-κB family of DNA-binding proteins; however, numerous routes for gene expression are likely deployed by discrete macrophage subpopulations dependent on their repertoire of receptors and signaling cascades. Engagement of membrane CD23 on human macrophages by IgE complexes stimulates expression of iNOS (Paul-Eugene et al. 1995). Glucocorticoids, transforming growth factor (TGF)-β, IL-4, IL-10 and IL-13 act to negatively regulate iNOS at the levels of transcription and mRNA stability (Doyle et al. 1994; Vodovotz 1997; Diaz-Guerra et al. 1999).

While numerous factors have been found to regulate transcription, deciphering mechanisms for posttranslational control of iNOS remains more elusive. Relative to the constitutive isoforms, iNOS is less susceptible to feedback inhibition from NO reaction with the heme moiety. The factors that control the iNOS subunit dimerization are an important determinant in the biochemistry of the enzyme (Venema et al. 1997; Stuehr 1999; Chen et al. 2002). However, the dynamics of iNOS monomer and dimer pools that likely exist within macrophages have not been determined (Baek et al. 1993). L-Arginine transport and intracellular metabolism are important regulators of NO biosynthesis amenable to pharmacological exploitation (Morris 1999; Closs et al. 2000). Uptake of L-arginine via the cationic amino acid transport systems are a rate-limiting factor for sustained iNOS activity (Hibbs et al. 1987a,b; Bogel et al. 1992). L-Arginine is

subsequently mobilized within macrophages differentially dependent upon the stimulant conditions (Morris et al. 1998). Concomitant changes in argininosuccinate synthase, arginase isoforms and iNOS expression upon activation point to the complexity of L-arginine metabolic cycles in macrophages. An intriguing feedback loop is the inhibitory action of N^{ω}-hydroxy-L-arginine, the initial oxidation product of L-arginine formed in the iNOS catalytic cycle (Eq. 5), on arginase (Chenais et al. 1993). *Helicobacter pylori* has evolved a survival mechanism involving inhibition of NO production by activated macrophages by usurping L-arginine availability through constitutive bacterial arginase activity (Gobert et al. 2001).

The fate of L-arginine in macrophages is controlled in part by distinct subcellular compartmentalization of enzymes and substrate pools. For instance, arginase I resides in the cytosol, while arginase II is present in the mitochondrial matrix (Morris 1999). The localization of iNOS, particularly in association with cytoskeletal elements (Webb et al. 2001; Zeng and Morrison 2001), may also play a prominent role in the effector functions of NO and related nitrogen oxides derived from macrophages (Vodovotz et al. 1995; Espey et al. 2001b).

3.2 RNOS and ROS Interactions

3.2.1 NO and O_{2-}

Peroxynitrite is a highly reactive RNOS formed by the reaction between NO and O_2^- (Eq. 7; Koppenol et al. 1992; Crow and Beckman 1995; Pryor and Squadrito 1995; Greenacre and Ischiropoulos 2001; Radi et al. 2001).

$$NO + O_2^- \rightarrow ONOO^- \tag{7}$$

Selective chemical modifications are observed upon exposure of many biological substances to $ONOO^-$ in vitro. Dependent on the constituents of the target, $ONOO^-$ can mediate both one- and two-electron oxidation as well as nitration reactions. Nitration involves the electrophilic addition of a NO_2^+ equivalent (nitronium, an electron acceptor) to a site of electron density, such as an aromatic ring. Transition metal catalysts and physiological levels of carbon dioxide augment nitration and oxidation yields derived from $ONOO^-$ (Lymar et al. 1996; Denicola et al. 1995, 1996). Development of antibodies that recognize nitrated tyrosyl residues (3-nitrotyrosine) has revealed the extent of nitration under a variety of disease conditions (Ye et al. 1996; Greenacre and Ischiropoulos 2001; Radi et al. 2001).

Caution should be exercised when attributing the cytotoxic actions of activated macrophages expressing iNOS to $ONOO^-$ formation (Ischiropoulos et al. 1992). The 10^9 M^{-1} s^{-1} rate constant for the $NO+O_2^-$ reaction signifies that these species will react with each other at near diffusion control (Huie and Padmaja 1993; Kissner et al. 1997). The stoichiometry of the reactants must also be taken

into consideration. Maximal oxidation and nitration mediated by $ONOO^-$ are observed when the rate of NO synthesis is equivalent to the rate of O_2^- production. A tip in balance in favor of either reactant results in a rapid decline or abatement of oxidation and nitration due to reactions secondary to $ONOO^-$ formation; for instance, reaction between ONOOH and NO (Rubbo et al. 1994; Miles et al. 1996; Wink 1997; Jourd'heuil et al. 1999; Espey 2002b,c). In general, exposure to $ONOO^-$ does not elicit cytotoxicity until relatively high bolus concentrations (>200 μM) are applied (Zhu et al. 1992; Espey et al. 2002d). Therefore, a prominent role for $ONOO^-$ as a macrophage effector RNOS would require a substantial and prolonged synthesis of both NO and O_2^- at equivalent rates.

Synchronization of both NO and O_{2-} rates of formation during an immune response would require a precise regulation to produce significant $ONOO^-$. Kinetic modeling of macrophages predict that the limited diffusion of O_2^- will restrict migration of $ONOO^-$ as an effector RNOS (Chen and Deen 2001). Assembly and translocation of the NADPH oxidase complex is a rapid process relative to de novo iNOS expression and protein synthesis, which fosters a temporal disassociation between O_2^- and NO production within an individual activated macrophage (Iyengar et al. 1987; Doyle et al. 1994; Espey et al. 2000b; Pfeiffer et al. 2001). This dichotomy gives rise to either ROS or RNOS formation during distinct phases of macrophage phagocytosis and immune responses, largely exclusive of $ONOO^-$ generation (Vazquez-Torres et al. 2000; Mastroeni et al. 2000; Espey et al. 2000b, 2002b; Pfeiffer et al. 2001). However, numerous studies have suggested that formation of $ONOO^-$ and derived intermediates are essential for killing pathogens in macrophage phagosomes or cytoplasm (Saran and Bors 1994; Nozaki et al. 1997; Linares et al. 2001; Hickman-Davis 2002). These reports are hampered by the lack of pharmacology or analytical methodology specific for $ONOO^-$ versus other ROS and RNOS pathways.

Innate immunity is dependent upon coordination between neutrophil and macrophage populations. Neutrophil infiltration characteristically precedes monocyte recruitment or activation of resident macrophages during acute inflammatory reactions (Metchnikoff 1905). This sequence results in an initial predominance of ROS generation by neutrophils at inflammatory foci. The ROS phase is downregulated by subsequent iNOS catalyzed formation of NO within activated macrophages. RNOS derived from NO autoxidation (see "NO and O_2" below) interfere with assembly of the neutrophil NADPH oxidase complex (Clancy et al. 1992; Fujii et al. 1997; Lee et al. 2000) and diapedesis of neutrophils through the vasculature (Kubes et al. 1991; Granger and Kubes 1994). In this manner, coincident generation of O_2^- and NO by neutrophils and macrophages, respectively, are spatially and temporally segregated minimizing the probability of $ONOO^-$ formation. A similar reciprocal relationship between endothelial NAD(P)H oxidase activation and eNOS in hemodynamic shear stress-induced monocyte chemotactic protein expression has been described (Wung et al. 2001). These data illustrate that $ONOO^-$ may be implicated only within discrete zones where conditions are ideal for contemporaneous and equivalent

rates of NO and O_2^- formation. It should be emphasized that NO is a highly effective ROS scavenger (Wink et al. 1995, 2001; Espey et al. 2002d).

A challenge for effective pharmacological intervention of iNOS in macrophages is to identify the timing for specific RNOS participation in the different phases of innate and adaptive immune responses. Although RNOS can compromise host cellular functions, they also play pivotal roles in both eradication of pathogens and abatement of ROS bystander injury.

3.2.2 NO and H_2O_2

Bacteria are rich in proteins containing Fe and Cu that facilitate electron transfer reactions (Salerno 1996). NO can disrupt these proteins (e.g., Fe-sulfur center of ferredoxins) causing the release of redox active metal ions (Drapier 1997; Poole and Hughes 2000). As described above ("Haber-Weiss Cycle"), the bacterial and mammalian cytotoxicity of H_2O_2 involves reduction by either Fe^{2+} or Cu^{1+} to yield either hypervalent metal ions or $^{\cdot}OH$ (Fenton 1894; Clifford and Repine 1982; Halliwell and Gutteridge 1984, 1999). NO-mediated labialization of metalloproteins in *Escherichia coli* bacteria has been shown to potentiate H_2O_2 toxicity more than 1,000-fold (Pacelli et al. 1995). Mobilization of transition metals from the periplasmic space and inner membrane to the genome was evidenced by DNA double-strand breaks.

Catalase is a heme-containing enzyme that catalyzes the catabolism of H_2O_2 into H_2O and O_2 (Chance 1947). NO can limit H_2O_2 catabolism through formation of nitrosylheme-catalase (Brown 1995; Brunelli et al. 2001). This may serve to augment the synergistic bactericidal action of NO and H_2O_2, provided NO is not overtly consumed by excess catalase. Paradoxically, other studies have suggested that virulence may be related to SOD-catalyzed conversion of O_2^- to H_2O_2 (De Groote et al. 1997). Periplasmic Cu,Zn-SOD may protect pathogenic bacteria, such as *E. coli* and *Salmonella typhimurium*, from redox injury (Benov et al. 1995; De Groote et al. 1997). Salmonella deficient in this Cu,Zn-SOD have reduced survival in macrophages and attenuated virulence in mice. These data illustrate that the tandem bactericidal action NO, O_2^- and H_2O_2 are dependent on a complex interrelationship between both host and bacterial systems that interact with ROS and RNOS.

Synergism between NO and H_2O_2 is not limited to bactericidal processes. NO formation by activated macrophages can disrupt transition metal homeostasis in tumor cell targets as well (Hibbs et al. 1984). Viability of a human ovarian cancer cell line exposed to 3-morpholino-sydnonimine, a compound that decomposes to simultaneously form NO and O_2^-, was found to not involve $ONOO^-$ formation. Rather, the mechanism of cytotoxicity was dependent NO and H_2O_2-mediated reduction of trace metals and generation of potent oxidants (e.g., $^{\cdot}OH$; see "Haber-Weiss Cycle" above; Farias-Eisner et al. 1996). In contrast to *Salmonella* and Cu,Zn-SOD, it was found that these cancer cells rely on the

glutathione peroxidase-glutathione reductase for H_2O_2 resistance and potential toxicity synergism with NO and reduced metals.

3.2.3
NO_2^- and H_2O_2

Nitrite is an end product of NO autoxidation (Eq. 8; see section "NO and O_2" below; Wink et al. 1993, 2000; Fukuto 1995).

$$4NO + O_2 \rightarrow 2N_2O_3 \rightarrow 4H^+ + 4NO_2^- \quad (8)$$

Marked increases in NO_2^- are observed during immune activation, which correlates well with iNOS induction in macrophages (Stuehr and Marletta 1985; Hibbs et al. 1987a,b; Granger et al. 1991, 1996; Ignarro et al. 1993; Lewis et al. 1995; Grisham et al. 1996; Espey et al. 2000b). Nitrite can be converted to NO_3^- by oxyhemoproteins (Rodkey 1976; Doyle et al. 1985; Ignarro et al. 1993) suggesting that the parenchymal concentration of NO_2^- at sites of inflammation may be much higher than the low micromolar levels (Miranda et al. 2001) detected in plasma. As mentioned above (in "Myeloperoxidase" section), MPO in the presence of H_2O_2 readily catalyzes oxidation of NO_2^- to generate NO_2 (Eq. 9 ; Sampson et al. 1995; Eiserich et al. 1996; van der Vliet et al. 1997; Hazen et al. 1999; Abu-Soud and Hazen 2000; van Dalen et al. 2000; Espey et al. 2002d).

$$2NO_2^- + H_2O_2 + 2H^+ \rightarrow 2NO_2 + 2H_2O \quad (9)$$

Similar to $ONOO^-$, NO_2 is an oxidant and nitrating agent. However, NO_2 produced by MPO/H_2O_2 and NO_2^- elicited substantially greater nitration than an equivalent amount of $ONOO^-$ generated from xanthine oxidase/hypoxanthine (an O_2^- source) and NO (Espey et al. 2002d). Differences in nitration were most pronounced under conditions that required transmembrane diffusion of the RNOS into target cells, consistent with data showing a significant lifetime for NO_2 (Espey et al. 2001, 2002d). Catalytic production of NO_2 by the MPO pathway is much less restricted by the spatial and temporal constraints on $ONOO^-$ formation in that all the components do not need to be generated contemporaneously. Nitration mediated by the MPO/H_2O_2 route may predominate subsequent to macrophage expression of iNOS and NO_2^- accumulation in lesion sites. Under these conditions, cellular or free MPO deposits in combination with episodic formation of H_2O_2 may lead to NO_2 generation over an extended period, which may play a role during inflammatory flares and chronic disease settings. In addition to MPO, protoporphyrin IX (hemin) in the presence of H_2O_2 catalyzes NO_2^- oxidation to form NO_2 resulting in nitration of tyrosyl-containing peptides (Thomas et al. 2002). NO production by activated macrophages may dislodge hemin from hemeproteins and generate copious amounts of NO_2^-. These low molecular weight agents may then move to sites involved in NADPH oxidase activity and H_2O_2 formation causing subsequent nitrative modifications via formation of NO_2.

3.2.4
NO and O_2

In the presence of O_2, the lifetime of NO is inversely proportional to its concentration due to the second order dependency of the autoxidation reaction on NO, which is third order overall (Eq. 10; Ford et al. 1993ε; Wink et al. 1993, 2000; Fukuto 1995).

$$-d[NO]/dt = k_{NO}[NO]^2[O_2] \quad (10)$$

The rate of NO autoxidation is influenced by the solubility of both NO and O_2 (Liu et al. 1998). Both species partition to the hydrophobic phase of membranes (Shaw and Vosper 1977; Subczynski and Hyde 1983), which accelerates the rate of NO autoxidation approximately 300-fold relative to the surrounding aqueous phase (Liu et al. 1998). The rate-limiting step for NO autoxidation in hydrophobic medium is the formation of NO_2, which subsequently reacts with NO to form equilibrium with N_2O_3 (Eqs. 11 and 12; Wink et al. 1993, 2000; Ford et al. 1993; Fukuto 1995).

$$2NO + O_2 \rightarrow 2NO_2 \quad (11)$$

$$NO_2 + NO \leftrightarrow N_2O_3 \quad (12)$$

Indeed, the autoxidation process within the architecture of intact cells was found to be distinct from that which occurs in the aqueous extracellular medium, suggesting that cellular hydrophobic domains in conjunction with scavenger composition and location serve to focus RNOS chemistry to discrete sites during NO formation (Espey et al. 2001b) and emphasize the importance of NO_2 intermediacy as a determinant in the functional outcome of NO biosynthesis (Espey et al. 2002b,c,d).

NO autoxidation becomes more competitive with other NO reaction pathways (e.g., NO+oxyhemeproteins) under conditions of high NO biosynthesis because the rate of autoxidation is proportional to the square of the NO concentration. Consistent with this, macrophages expressing iNOS have been shown to form NO adducts on a variety of nucleophiles via formation of N_2O_3 (Miwa et al. 1987; Iyengar et al. 1987; Kosaka et al. 1989; Wink et al. 1997, 2000; Espey et al. 2001a,b). This process is termed nitrosation, where N_2O_3 acts as a nitrosonium donor (NO^+; Williams 1988). Nitrosation has been implicated in the modification of a variety of proteins containing critical thiol, amine, and hydroxyl residues (Wink et al. 2000; Espey et al. 2000a, 2001). Of particular interest, N_2O_3 formation and nitrosative facility of macrophages was dependent on the route of activation (Espey et al. 2000b). Macrophages stimulated with IFN-γ and LPS contained a twofold greater iNOS protein level compared to macrophages activated with IFN-γ and either TNF-α or IL-1β; however, the difference in nitrosative capacity was greater than 30-fold. These data show that the NO profiles derived from iNOS can be distinct and depend on inductive signal cascades. Formation of NO adducts on amines and thiols can occur during concurrent

O_2^- and NO biosynthesis through either N_2O_3-mediated nitrosation or oxidative nitrosylation (e.g., thyl radical+NO→RSNO) further demonstrating the complex interrelationships between ROS and RNOS and the functional outcomes of macrophage activation (Espey et al. 2002c).

4 Oxygen

An understanding of the dynamic changes in molecular O_2 usage is crucial to deciphering macrophage ROS and RNOS cascades. On the basis of changes in electron paramagnetic resonance spectroscopy signals from spin labels DEPMPO (for O_2^-) and PDT (for O_2), O_2^- generation from activated neutrophils corresponded to 50% of oxygen uptake (Roubaud et al. 1998). Metabolic labeling studies suggest that consumption of O_2 also increases upon stimulation in macrophages, but a smaller percentage is reduced to O_2^- (Baehner and Johnston 1972; Baehner et al. 1975; Reiss and Roos 1978). In severely hypoxic environments, it is possible that oxygen tension may be insufficient to sustain O_2^- formation (Edwards and Lloyd 1988). However, a rebound phenomenon of augmented O_2^- generation may occur upon re-oxygenation (Wilhelm et al. 1997). Generation of N_2O_3 by activated macrophages can nitrosate intracellular glutathione to form *S-nitrosoglutathione* thereby activating bioreductive metabolism via the hexose-monophosphate pathway and glutathione reductase (Albina and Mastrofrancesco 1993; Clancy et al. 1994). Additional changes in O_2 usage by activated macrophages and surrounding cells is manifest by the reversible inhibition of mitochondrial respiration by NO, which has a greater affinity for cytochrome oxidase than O_2 (Brown 1997, 2000; Boveris and Poderoso 2000). NO also interacts within macrophages to stabilize hypoxia-inducible factor and modulate its associated transcription networks (Brüne et al. 2001; Sandau et al. 2001).

5 Conclusion

These examples underlie the concept that participation of ROS and RNOS in disease processes is highly dependent on the state of tissue. Parameters, such as pO_2, may change quickly as waves of different leukocyte populations are engaged within inflammatory lesions over a period of time, subsequently affecting the levels and types of reactive intermediates formed. The action of ROS and RNOS are diverse and dependent on their rates of formation, mobility, interaction with each other, and the composition of the surrounding milieu. Coordination of macrophage-derived ROS and RNOS formation involves signaling from soluble, cell–cell, and matrix cues. These pathways vary greatly among the specialized members of the macrophage lineage. Macrophages have numerous mechanisms that control and balance formation of specific ROS and RNOS toward combating pathogens and preserving host tissue. With an increased

awareness of feedback mechanisms, pharmacological strategies aimed at alleviating macrophage-mediated cellular stress and injury will improve.

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Proteases

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Abstract The macrophage, as a gatekeeper to both the innate and acquired immune systems, has great potential as a therapeutic target for such diverse human disease states as bacterial and viral infection, autoimmunity, inflammatory diseases, and cancer. The phenotype of macrophages in different tissues varies markedly between tissues. While this characteristic creates technical challenges in terms of isolation and characterization of resident tissue macrophages, it opens the possibility of targeting individual tissue-specific macrophage populations for pharmacologic intervention. The proteases are among the most numerous and abundant of enzyme classes, representing 1%–4% of all proteins encoded by eukaryotic genomes. Proteases are particularly abundant in macrophages, where they are critical players in many key functions of the macro-

phage, such as degradation of exogenous, potentially pathogenic proteins; digestion of both foreign and self proteins into peptides for presentation by MHC class I and II; and functional regulation of target proteins, for example by removal of a regulatory domain or a transmembrane anchor. This chapter reviews some of the proteases expressed in macrophages, and discusses what functional roles have been shown for, or postulated for, these enzymes. The enzymes discussed here are divided into two main groups: ectoproteases, which cleave amino acids from either end of a protein or peptide, and endoproteases, which cleave proteins at internal sites. Examples are given illustrating the actions of proteases within the macrophage, at the cell surface, and after secretion into the extracellular milieu.

Keywords Aminopeptidase, Angiotensin converting enzyme, Carboxypeptidase, Caspase, Cathepsin, CPVL, Matrix metalloprotease, TNF-? converting enzyme

1 Introduction

The role of macrophages lies at the interface of the innate and adaptive immune systems. Innate immune functions include phagocytosis of both unopsonized and opsonized pathogens, release of toxic free radicals, and secretion of inflammatory mediators such as cytokines, chemokines, and a large variety of other extracellular signaling molecules. Within the adaptive immune response, macrophages process and present antigen to T cells, and are capable of providing both immunogenic and tolerogenic signaling through secretion of cytokines and other soluble mediators. This unique immunomodulatory role of macrophages makes them ideal candidates for pharmacological intervention, with the potential to treat a highly diverse set of human diseases, including bacterial and viral infections, autoimmunity, inflammatory conditions, and cancer. An important hallmark of macrophages is their ability to adapt to their cellular surroundings, leading to extreme phenotypic diversity of macrophages. While this diversity presents the researcher with certain challenges, it also represents a unique opportunity for pharmacological intervention. If the nature of this diversity is understood, it may be possible to treat restricted subsets of macrophages without affecting others, thus greatly increasing drug specificity.

The proteases are among the most numerous and abundant of enzyme classes. The MEROPS database (Rawlings et al. 2002) (http://www.merops.sanger.ac.uk) lists nearly 400 different human proteases for which a chromosomal location has been mapped, representing more than 1% of the human genome. Analysis of the more completely characterized eukaryotic genomes has shown that proteases comprise between 1.7% and 3.9% of expressed genes, suggesting the presence of a large number of unknown or poorly characterized human proteases. Proteases can be subdivided functionally into those that can cleave internal polypeptide sequences (endopeptidases) and those that cleave only from

one end of the substrate molecule (ectopeptidases). The ectopeptidases are further divided into enzymes cleaving from the N and C termini, called aminopeptidases and carboxypeptidases, respectively. Proteases perform a wide variety of physiological functions throughout the body, both inside and outside the cell. From a pharmacological standpoint, a comprehensive understanding of protease function is critical, both for identification of new drug targets and to anticipate and ameliorate the side effects associated with protease inhibition. Known protease functions include: (1) intracellular destruction of proteins that are senescent, misfolded, or expressed cyclically; (2) breakdown of foreign, potentially pathogenic proteins; (3) digestion of both foreign and self proteins into peptides for presentation by MHC class I and II; (4) activation and execution of cell death cascades, acting either on itself or on an adjacent target cell; (5) functional activation and/or inactivation of enzymes, bioactive peptides, and many other types of proteins by proteolytic removal of a regulatory domain; (6) release of proteins from the plasma membrane or other membrane-bound compartment by cleavage of a transmembrane domain; and (7) digestion of proteins in the digestive tract for nutritional purposes.

Since the vast array of proteases in the human body display widely variable specificities and inhibitor sensitivities, the value of proteolysis as a pharmacologic target is clear. Targeting proteolytic pathways has led to such drug successes as angiotensin converting enzyme inhibitors for high blood pressure (Douglas 1985) (see below), the plasmin-targeted thrombolytic agents such as streptokinase and urokinase (Reilly 1985), and HIV protease inhibitors as part of combination therapy for HIV infection (Hammer et al. 1997; Gulick et al. 1997). Proteases are particularly abundant in macrophages, where they perform a wide variety of functions. These functions, which will be described in more detail below, include destruction of phagocytosed material, trimming of peptides for presentation by MHC class II molecules, alterations of extracellular matrix components, and a variety of regulatory roles. This chapter will provide an overview of the properties of proteases found in macrophages, and will attempt to highlight some areas of interest for further basic biochemical study and potential pharmacologic intervention.

2 Ectoproteases

Ectoproteases differ from the more abundant endoproteases in that they cleave substrates only at the carboxy or amino terminus. This functional difference has a structural basis: the substrate binding sites of ectoproteases tend to be solvent-accessible at only one end, thus allowing cleavage at the end of a protein but not in the middle of a polypeptide loop. This difference has important implications in design of synthetic inhibitors. Properties of ectoproteases found in macrophages are discussed below. Alternate names are shown in parentheses.

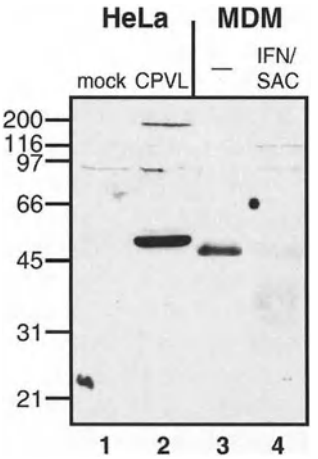

Fig. 1 Regulation of CPVL expression by macrophage activation state. Polyclonal anti-CPVL Western blot of lysates from mock- and CPVL-transfected HeLa cells (*lanes 1 and 2*) shows a major specific band of about 48 kDa. Human monocyte-derived macrophages (*MDM, lanes 3 and 4*) were cultured for approximately 10 days in RPMI 1640 plus 10% fetal bovine serum, either alone or with IFN-γ and *Staphylococcus aureus* cells (*SAC*, a source of lipopolysaccharide). Activation led to complete loss of the immunoreactive band. Equal protein loading was confirmed by Ponceau S staining (not shown). Note that recombinant CPVL migrates slightly more slowly because of an N-terminal epitope tag

2.1 Carboxypeptidase, Vitellogenic-Like (CPVL, CP-Mac)

CPVL (MEROPS ID S10.003) is a 476 amino acid serine carboxypeptidase, discovered as a result of a differential display polymerase chain reaction (PCR) screen for novel macrophage-specific genes (Mahoney et al. 2001). RT-PCR, Northern blot, and Western blot analysis confirm that, among hematopoietic cells, CPVL is indeed restricted to the monocytic lineage. CPVL mRNA was readily detected throughout the lineage, whereas protein expression was absent or low in monocytes and relatively abundant in mature monocyte-derived macrophages. Outside the immune system, however, a wider expression pattern was apparent. High levels of expression, as judged by Northern blot, were apparent in kidney and heart, two organs with few macrophages, while lung and liver, which have much larger macrophage populations, expressed little or no CPVL mRNA. We postulate that CPVL has two distinct expression profiles, one in a subset of tissue macrophages and a second one, presumably representing a separate function, in organs of the cardiovascular system. This pattern is similar to the expression pattern of angiotensin-converting enzyme (ACE, see below), which also shows significant expression in macrophages, heart, and kidney, although ACE is detectable in virtually all organs because of its presence in vascular endothelial cells (Dzau et al. 2001). Moreover, an alternate isoform of ACE is highly expressed in testis. Surveys of the human expressed sequence tags (EST) database suggest that CPVL is also expressed in testis.

The physiological function of CPVL is not currently known. Both primary sequence analysis and pulse-chase experiments (unpublished studies) suggest a lumenal and/or secreted distribution. In preliminary immunocytochemical studies using confocal microscopy, CPVL showed cytoplasmic expression in a vesicular pattern that did not coincide with endosomes, lysosomes, or MHC class II peptide-loading compartments (B. Ntolosi, R. DaSilva, and S. Gordon,

personal communication). Protein levels of CPVL are strikingly modulated by inflammatory stimuli: Culture of developing macrophages in the presence of interferon (IFN)-γ and lipopolysaccharide causes a dramatic downregulation of cellular CPVL expression (Fig. 1). While a great deal more work is required to ascertain the importance of this macrophage-restricted protease, we speculate that CPVL may play a role in the macrophage inflammatory response.

2.2 Carboxypeptidase M (CPM)

CPM (MEROPS ID M14.006) is a 439-amino acid glycosylphosphatidyl inositol-linked, membrane-bound metallo-carboxypeptidase (Tan et al. 1989) with preference for removal of lysine or arginine. While it is found in several different cell types, it is strongly expressed in monocytic lineage cells. Just as is the case with CPVL, CPM expression is upregulated during maturation of monocytes to macrophages (Rehli et al. 1995). CPM cleaves the C-terminal basic amino acid from a variety of biologically active peptide substrates, including bradykinin, dynorphin A(1–13), and enkephalins (Skidgel et al. 1989). It has been suggested, though not yet explicitly shown, that CPM would cleave and thus inactivate the anaphylatoxins C3a/C4a/C5a, in the same way as the related liver enzyme carboxypeptidase N (Rehli et al. 2000).

The level of CPM expression in vivo is highly dependent on the activation and/or differentiation state of the cells. Expression in macrophages of secondary lymph organs is low, but macrophage CPM expression during rejection of kidney transplants is much higher, and this elevated expression is inhibited by cyclosporin treatment (Andreesen et al. 1988). Moreover, monocytes from patients with aplastic anemia (Andreesen et al. 1989) or HIV (Andreesen et al. 1990) do not display maturation-induced CPM upregulation, even when cultured with healthy serum. These data suggest that CPM activation may be part of a macrophage inflammatory process. While these types of data show a compelling correlation between CPM levels and macrophage activation, proof of the importance of CPM in macrophage activation will require more understanding of its physiologically relevant substrates.

2.3 CD13 (Aminopeptidase N, Alanine aminopeptidase)

CD13 (MEROPS ID M01.001) is, like CPM, a plasma membrane-bound ectoprotease, consisting of 967 amino acids and one N-terminal transmembrane domain (Olsen et al. 1988). CD13 is ubiquitously expressed, and highly expressed in monocytic and granulocytic cells. As an aminopeptidase, CD13 removes single amino acids from the N termini of proteins and peptides. Like most ectopeptidases, CD13 is capable of removing amino acids from small bioactive peptides. Strikingly, removal of a single amino terminal residue from the chemokine monocyte chemotactic protein (MCP)-1 converts this basophil-activating che-

mokine into an eosinophil-activating one (Weber et al. 1996), taking advantage of the alternate expression of chemokine receptors with overlapping specificity in these cell types. However, its primary role appears to be the trimming of antigenic peptides bound to MHC molecules (Larsen et al. 1996). Therefore, the relative efficiency of CD13 to perform this function on antigen-presenting cells may significantly affect the balance of epitopes presented to T cells, with important ramifications in autoimmunity.

In addition to these physiologic roles, CD13 is also the receptor for coronaviruses to attach to endothelial cells of the upper respiratory tract (Yeager et al. 1992). Cytomegalovirus also uses CD13 as its receptor, as evidenced by the inhibition of both viral binding and infection with anti-CD13 antibodies in vitro (Soderberg et al. 1993). Cytomegalovirus binding to targets can lead to production of chronic graft-versus-host disease and pathogenic anti-CD13 autoantibodies (Soderberg et al. 1996).

2.4 Lysosomal Protective Protein (LPP, Cathepsin A, PPCA, Lysosomal Carboxypeptidase A)

LPP (MEROPS ID S10.002) is a lysosomally localized serine carboxypeptidase that, along with CPVL and a smooth muscle cell protein called RISC (Chen et al. 2001), make up the only three serine carboxypeptidases known in mammals. LPP was first isolated as the gene mutated in the human lysosomal storage disease galactosialidosis (Galjart et al. 1988), a syndrome caused by instability of lysosomal beta galactosidase and neuraminidase in the absence of a 54-kDa "protective protein," which normally protects these enzymes from degradation in the harsh lysosomal environment. Phenotypes vary by exact mutation, but generally include dwarfism, mental retardation, and a macular cherry-red spot. Sequence analysis showed similarity to serine proteases, and serine carboxypeptidase activity was soon confirmed but, importantly, shown not to be required for the protective function (Galjart et al. 1991).

LPP has three distinct enzymatic activities: an esterase activity, a deamidase activity, and a carboxypeptidase activity (Jackman et al. 1990). The protein is made as a 452 amino acid, 54-kDa precursor, which is then cleaved into 32-kDa and 20-kDa subunits (Pshezhetsky 1998). Mature LPP cleaves a variety of bioactive peptides in vitro, including bradykinin, endothelin I, substance P, and oxytocin (Jackman et al. 1990; Pshezhetsky 1998). However, the physiologically relevant substrates are not known.

Significant progress has recently been made in the treatment of a galactosialidosis model disease in mice (LPP knockout mice), which raises the possibility that this disease may be treatable in humans. D'Azzo and colleagues transplanted LPP knockout mice with bone marrow cells transduced to overexpress LPP under the influence of the colony stimulating factor-1 promoter, which directs expression to monocytes and macrophages. Since bone marrow-derived monocytic lineage cells traffic to essentially all tissues and secrete LPP, resident cells

may take up the secreted LPP, thus curing the defect in their own lysosomes. The treated mice showed marked reduction in symptoms and histopathology, with virtually all but the loss of cerebellar Purkinje cells corrected (Hahn et al. 1998). Their most recent model used murine stem cell virus to stably infect bone marrow cells with LPP and green fluorescent protein from a bicistronic vector (Leimig et al. 2002). The treated mice showed marked improvement for many months, including sparing of Purkinje cells. As this impressive work continues on the protective role LPP and its link to galactosialidosis, little information has emerged so far on the physiological role of the enzyme activities of this protein. Lysosomal storage disorders are discussed in detail in Chap. 11.

2.5
Angiotensin Converting Enzyme (ACE, Peptidyl Dipeptidase, Kininase II)

ACE (MEROPS ID X06.001) is a 1,306 amino acid cell surface bound protein containing two independent metalloprotease domains (Soubrier et al. 1988). It is widely expressed in somatic cells, and an alternate form with only the C-terminal protease domain is expressed only in male germ cells. ACE is a dicarboxypeptidase, cleaving two amino acids from the C terminus of the inactive angiotensin I, thus creating the powerful vasoconstrictor angiotensin II. Inhibitors of ACE such as captopril, enalapril, and numerous others have been extremely valuable agents for controlling hypertension (Douglas 1985).

Macrophages express high levels of ACE, and several reports within the past few years have emphasized the pathophysiological importance of macrophage ACE. It has long been known that ACE inhibition is beneficial in the treatment of atherosclerosis. Diet et al. showed that ACE accumulates in atherosclerotic plaques, and that the main source of ACE is foam cells, the characteristic lipid-laden macrophages of atherosclerosis (Diet et al. 1996). Moreover, they showed that differentiation of the monocytic cell line THP-1 into a macrophage phenotype led to an increase in ACE activity, and that increase was potentiated by addition of acetylated LDL. The mechanism of this effect is unknown, but probably involves inhibition of inflammatory mediators such as MCP-1 and interleukin (IL)-12 in the macrophages (Hernandez-Presa et al. 1997; Constantinescu et al. 1998). Finally, treatment of human mononuclear cells with ACE inhibitors in vitro decreased the synthesis of tissue factor, the clotting cascade initiator implicated in arterial thrombosis (Napoleone et al. 2000).

3
Endoproteases

3.1
Cathepsins

The term cathepsins does not refer to a group of proteins related by evolution, but rather by location and function. Cathepsins are a group of lysosomal pro-

teases, most of which are involved in the degradation of phagocytosed or endocytosed products. They can be of any enzyme class, although most are cysteine proteases. Many of the cathepsins are ubiquitously expressed, but as the numbers of known cathepsins increases, some cell type-specific examples are emerging.

Cathepsins B and D (MEROPS IDs C01.060, A01.009) have been implicated in the degradation of apolipoproteins in macrophages (Kuroda et al. 1994). Apolipoproteins from oxidized LDL particles are not digested well by macrophages, potentially leading to accumulation of foam cell macrophages and atherosclerosis. This inhibition of apolipoprotein digestion appears to be mediated by oxidized LDL inhibition of cathepsin B, via an unknown mechanism (Hoppe et al. 1994).

Cathepsin K (MEROPS ID C01.036) is one of the most cell type-specific of this group. While cathepsin K was originally thought to be expressed only on osteoclasts, the macrophage lineage cells responsible for bone resorption (see Chap. 19, this volume), recent evidence suggests that macrophages involved in foreign body responses, such as multinucleated giant cells or epithelioid cells in granulomas, also express it (Buhling et al. 2001). Resident tissue macrophages did not express cathepsin K, whereas cathepsins B and L were expressed on both resident and foreign body-elicited macrophages.

A final example of macrophage-specific cathepsin function comes from the study of processing of the invariant chain, Ii, by antigen-presenting cells. The invariant chain associates with nascent MHC class II molecules, to prevent binding of endogenous antigens. When the class II molecule enters the endosomal compartment, Ii is cleaved by a cathepsin, leaving only the small class II-associated invariant-chain peptide (CLIP) in the MHC groove, to be exchanged for a newly processed antigenic peptide. Chapman and colleagues showed that cathepsin S (MEROPS ID C01.034) is required for Ii cleavage in B cells and dendritic cells (Shi et al. 1999). They went on to show that MHC class II presentation in macrophages of cathepsin S knockout mice was normal, and identified a novel protease, cathepsin F (MEROPS ID C01.018), responsible for this activity in macrophages (Shi et al. 2000).

3.2 Caspase-1 (Interleukin 1β Converting Enzyme, ICE)

Caspases are cysteine endoproteases that cleave after Asp residues, in the context of a four amino acid recognition motif, in a wide variety of protein substrates. Caspases are best known for their involvement in the apoptosis cascade (Earnshaw et al. 1999). However, a subset of caspases are primarily involved in proteolytic release of cytokine precursors from the membrane, for action at a distant site. The best-known example is caspase-1 (MEROPS ID C14.001), which was identified by its ability to release IL-1β from monocytes and macrophages (Thornberry et al. 1992). Mice deficient in caspase-1 cannot release IL-1β, and thus are resistant to septic shock, an IL-1β-dependent process (Li et al. 1995). It was subsequently shown that caspase-1 also catalyzes the release of IL-18, or in-

terferon γ inducing factor (Ghayur et al. 1997; Gu et al. 1997). These data raise the possibility that a specific inhibitor of caspase-1 may be a useful treatment for sepsis. In the meantime, this area has provided an explanation for the regulatory effect of nitric oxide on inflammatory cytokine release. Nitric oxide potently inhibits cysteine proteases by S-nitrosylation of the active site cysteine. Kim et al. showed that activated macrophages treated in vitro with a nitric oxide synthase inhibitor released fourfold more IL-1β than those untreated (Kim et al. 1998). Furthermore, mice deficient in inducible nitric oxide synthase produced more IL-1β and more interferon γ in response to challenge with endotoxin.

3.3 Proteases Secreted to Act on Their Environment

3.3.1 Macrophage Gelatinase (Matrix Metalloprotease 9, MMP9, Gelatinase B)

MMP9 (MEROPS ID M10.009) is a 707 amino acid zinc metalloprotease (Wilhelm et al. 1989) and member of the matrix metalloprotease (MMP) family, a large (>20 different genes discovered) family of zinc metalloproteases responsible for the clearance and remodeling of the extracellular matrix, with downstream effects in areas such as development and wound healing (Nagase and Woessner 1999). The MMPs are highly regulated by gene expression, by synthesis as inactive preproenzymes, and by the presence of inhibitors such as the tissue inhibitors of metalloproteases (TIMPs). Unlike some other groups of proteases, such as the cathepsins, MMPs are generally not expressed in normal tissue, but expression is induced by a variety of stimuli, such as cytokines, growth factors, and others.

MMP9 is expressed on macrophages and neutrophils, and like all gelatinases, degrades a variety of extracellular components such as collagens, elastin, and fibronectin. However, the physiological roles played by this enzyme (and the others in this section; see below) are surprisingly broad, as demonstrated by the results of knockout studies. MMP9 knockout mice showed defects in bone formation and vascularization, caused by a lack of MMP9 in chondroclasts, the multinucleated, bone marrow-derived cells that resorb cartilage (Vu et al. 1998). Other studies showed that MMP9 knockout mice had reduced capacity for outgrowth of oligodendrocyte processes in the developing brain (Oh et al. 1999), and diminished ability for exogenously implanted tumors to metastasize (Itoh et al. 1999). Intriguingly, the tumors did not express MMP9, but rather required MMP9 secreted from host cells for successful metastasis. Finally, MMP9 has been shown to cleave a short amino terminal peptide from the neutrophil chemokine IL-8, and this modified IL-8 was more than tenfold more potent in neutrophil activation and chemotaxis assays (Van den Steen et al. 2000). Therefore, MMP9 has pleiotropic effects because of its matrix proteolytic functions, and moreover, exerts immunostimulatory effects by modification of a chemokine.

3.3.2
Macrophage Metalloelastase (MME, MMP12)

MMP12 (MEROPS ID M10.009) is a 470 amino zinc metalloprotease (Shapiro et al. 1993) in the MMP family. MMP12 degrades elastin and other extracellular matrix components. Consistent with the notion that many MMPs are inducible proteins, MMP12 signal is only detected in placenta (a highly macrophage-enriched tissue) by Northern blotting (Belaaouaj et al. 1995). Experiments with MMP12 promoter constructs indicated that MMP gene expression was induced by LPS in a mouse macrophage cell line, but not in human umbilical vein endothelial cells. Knockout studies showed that MMP12 was necessary for matrix degradation by macrophages in vitro and in vivo. $MMP12^{-/-}$ macrophages had reduced proteolytic activity against insoluble elastin in vitro, and the ability to penetrate Matrigel artificial basement membranes in vitro and in vivo was abolished (Shipley et al. 1996). Strikingly, the knockout conferred complete protection in an experimental model of cigarette smoke-induced emphysema (Hautamaki et al. 1997). Control mice exposed to cigarette smoke for 3 months showed immunohistochemical evidence of MMP12-positive macrophage recruitment to the lungs, and increased mean alveolar air space. Neither effect was detectable with $MMP12^{-/-}$ mice. If macrophage recruitment of $MMP12^{-/-}$ macrophages was artificially induced by adding the chemokine MCP-1, the (MMP12-negative) macrophages were detected in the lung, but there was still no change in mean alveolar air space. These experiments suggest that an MMP12-specific inhibitor has potential therapeutic value in the setting of pathological macrophage recruitment.

3.3.3
Leukocyte Elastase (LE, Neutrophil Elastase)

LE (MEROPS ID S01.131), despite sharing many properties with MMPs 9 and 12, is not a member of the MMP family, but rather is a 218 amino acid serine endoprotease (Sinha et al. 1987). Although LE is expressed in macrophages, it is most highly expressed in neutrophils, where it can be the cause of destructive lung disease (Mitsuhashi et al. 1999). It was recently reported that cystic fibrosis patients have impaired removal of apoptotic inflammatory cells by macrophages. This defect is caused by LE-mediated cleavage of the phosphatidylserine receptor that recognizes apoptotic cells for uptake (Vandivier et al. 2002). The lost capacity to dispose of toxic mediators from apoptotic inflammatory cells may exacerbate the deleterious effects of these cells.

3.4
Proteases Acting at the Cell Surface

3.4.1
TNF-α Converting Enzyme (TACE, ADAM17, CD156b)

Tumor necrosis factor (TNF)-α is a powerful pro-inflammatory cytokine that, like IL-1β, is synthesized in a plasma membrane-bound form, and is then released into the extracellular space by TACE (MEROPS ID M12.217). TACE is an 824 amino acid member of the ADAM (the name is derived from a disintegrin and metalloprotease) family, a group of over 40 proteins containing a disintegrin domain that binds integrins and a metalloprotease domain similar to those in the MMP family (Black et al. 1997; Moss et al. 1997). While TACE is ubiquitously expressed, it is highly expressed in some macrophage populations, where TNF-α is made and secreted. Because TNF-α is often implicated in harmful inflammatory pathways, there has been great interest in devising methods to inactivate TACE. Methods for inhibiting TACE include use of the natural inhibitor TIMP-3 (Amour et al. 1998), creation of a recombinant dominant negative form of the enzyme (Solomon et al. 1999), and development of small molecule inhibitors (Barlaam et al. 1999).

Surprisingly, attempts to create a TACE knockout mouse led to the finding that TACE has a much broader substrate specificity. Mice carrying a mutation in the Zn binding site of TACE had a large number of developmental abnormalities, and most died between embryonic day 17.5 and 1 day after birth (Peschon et al. 1998). Their mutations were reminiscent of those in mice deficient in transforming growth factor (TGF)-α, which is also released from the plasma membrane by a cleavage event. The authors went on to show that TACE is responsible for cleavage of the ectodomains of TGF-α, L-selectin, and TNF receptor p75. Subsequent work by other groups has shown that other proteins of potential therapeutic significance are also cleaved by TACE, including the amyloid protein precursor associated with Alzheimer's disease (Buxbaum et al. 1998), and the receptor for colony stimulating factor-1, the factor critical for commitment of precursor cells to the monocytic lineage (Rovida et al. 2001).

3.4.2
Macrophage Mannose Receptor Secretase

Macrophage mannose receptor (MMR) is a 180-kDa glycoprotein expressed on the plasma membrane of macrophages, some dendritic cells, and a few isolated endothelial cell types (Linehan et al. 2000). Eight C-type lectin domains mediate its functions as a phagocytic and endocytic receptor, recognizing mannose- and fucose-containing structures. A second carbohydrate recognition domain, the N-terminal cysteine-rich (CR) domain, mediates binding to sulfated sugars on ligands such as sialoadhesin and CD45 expressed on marginal zone macrophages and in germinal centers (Martinez-Pomares et al. 1996; Martinez-

Pomares et al. 1999). MMR is released from macrophages in vitro and in vivo by a metalloprotease-type secretase (Martinez-Pomares et al. 1998). Martinez-Pomares and Gordon have proposed that MMR may transport polysaccharide antigens to secondary lymphoid organs for generation of immune responses (Martinez-Pomares and Gordon 1999). To date little is known about the nature of the MMR secretase, except that it is present on macrophages, and is susceptible to hydroxamate-based inhibitors. Given these facts, one may speculate that MMR secretase is, in fact, TACE. However, since most of the over 40 members of the ADAM family share the same basic domain structure as TACE, and as yet have no described function, there is no shortage of good candidates.

4 Summary

Proteases are critical players in many of the central functions of macrophages, including digestion of phagocytosed material, processing of foreign antigens for presentation on MHC class II molecules, tissue remodeling, and regulation of immune responses by activating, inactivating, or releasing from membranes a host of immune-active proteins and peptides. The task of understanding the roles of these enzymes is complicated by the fact that many proteases have multiple and overlapping functions. However, with the appropriate tools and insight, assisted by the arrival of whole genome data sets, the prospects for major advances in understanding the pathophysiology of this system are bright, leading to significant advancements in the treatment of human disease.

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Targeting the Chemokine System

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1 Introduction to the Chemokine System

The chemokine superfamily consists of small, basic, heparin-binding proteins that play a pivotal role in basal trafficking as well as in activation and recruitment of leukocytes from the circulation to sites of inflammation. The chemokines are a subset of the cytokine family and are distinguished from other cytokines in that they activate seven-transmembrane (7TM) G protein-coupled receptors. They are a large family, with approximately 50 members identified to date, and for which 19 receptors have been described. The chemokine family is divided structurally into four subfamilies CXC, CC, CX_3C and C, based on the position of the amino terminal cysteine residues. The majority of chemokines fall into the CXC or CC groups (also referred to as α and β subclasses respectively), and hence have been the most extensively studied. The known chemokine/receptor pairs are depicted in Fig. 1, which also indicates a second division based on the recent advances in chemokine biology—chemokines are either expressed constitutively and control basal trafficking or homing, or are inducible,

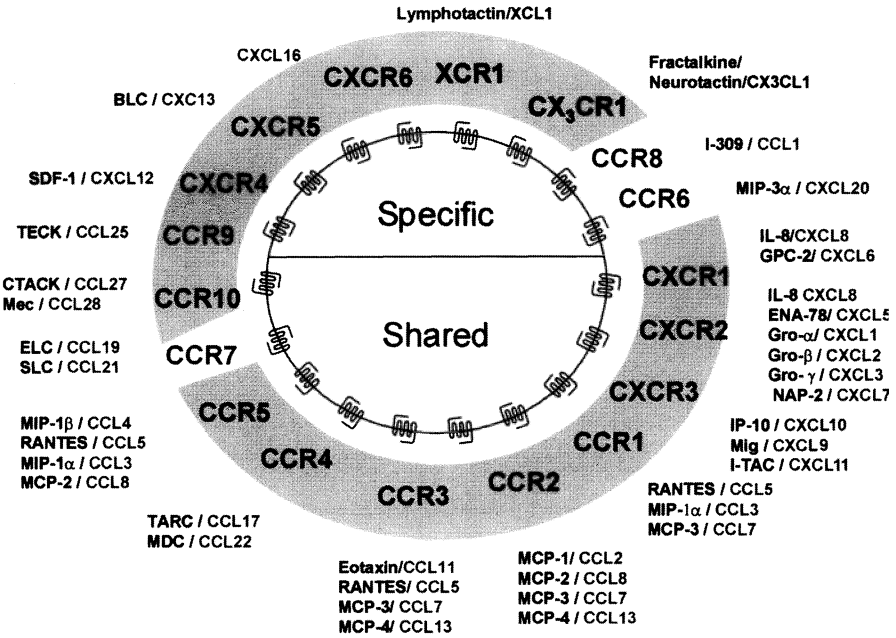

Fig. 1 Chemokine receptor–ligand pairing. The known chemokine receptor–ligand pairs are shown. Both the common names for the chemokines, as well as their systematic nomenclature are given. Receptors that bind a single ligand are classified as specific, while those that bind more than one ligand are classified as shared. Receptors which are constitutively expressed are shaded in *grey*, while those that are inducible are shaded in *orange*. This division must not be considered as absolute, since the receptors that are not shaded have overlapping properties, for example CCR6 is downregulated, while CCR7 is upregulated during the maturation process of dendritic cells, and CCR8 has been implicated in allergic inflammation, although it is expressed constitutively in the thymus. The main point to be made is that the receptors classified as inducible have been shown to play a role in inflammation

and are involved in inflammation. Chemokines were generally named according to the function that was identified such as monocyte chemoattractant protein (MCP) or neutrophil activating peptide (NAP) but since many chemokines were concomitantly identified in more than one laboratory, a single sequence was attributed more than one name. Therefore a systematic nomenclature was recently adopted (Zlotnik and Yoshie 2000) and both common and systematic names are shown in Fig. 1.

Chemokines do not necessarily have a high level of homology at the level of primary amino acid sequence, but they have a very highly conserved monomeric three-dimensional structural fold, which is conferred on them by the canonical 4-cysteine motif, which the majority possess. Their monomeric fold is superimposable for all chemokines whose structures have been solved to date (Fig. 2A), independent of the subfamily to which they belong. However, many chemokines form dimers, and the dimeric structure of CXC chemokines is very different from that of the CC chemokines as shown in Fig. 2. The CXC subclass

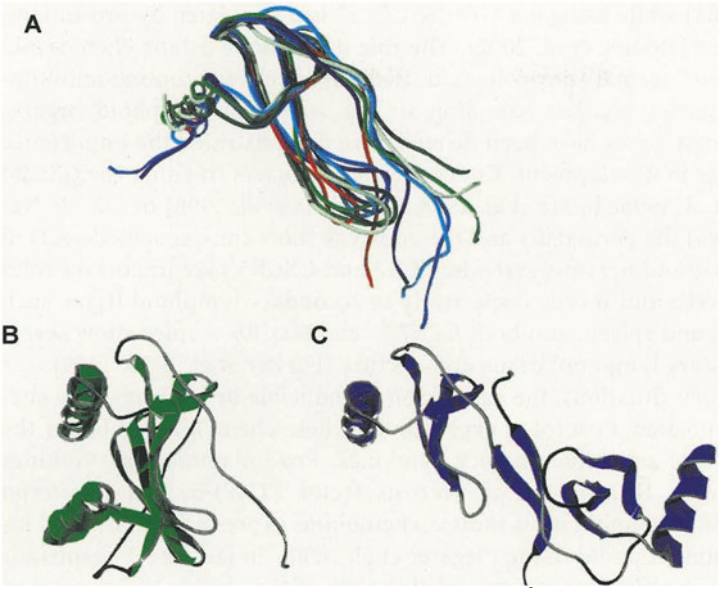

Fig. 2A–C The monomeric and dimeric topology of chemokines. **A** The superposition of seven monomeric chemokine structures. The CC chemokines are shown in *blue*: RANTES/CCL5, *dark blue*, MCP-1/CCL2, *medium blue* and MIP-1β, *light blue*; the CXC chemokines are shown in *green*: PF4/CXCL4, *dark green*, IL-8/CXCL8, *medium green* and NAP-2/CXCL7, *light green*, and the CX_3C chemokine, fractalkine in shown in *red*. **B** The dimeric structure of the CXC chemokine IL-8/CXCL8. **C** The dimeric structure of the CC chemokine RANTES/CCL5

form compact dimers, with the two carboxy helices lining a groove formed by 4 beta sheets, rather similar to the MHC class II groove, while the CC chemokines form elongated cylindrical dimers through interactions of their amino termini. It is widely debated whether the dimeric structure is in fact physiologically relevant, since dimers form at micromolar concentrations, whereas chemokines are active at nanomolar concentrations. While it is well documented that the monomeric forms of chemokines that are known to dimerize are fully active in vitro (Rajarathnam et al. 1994; Paavola et al. 1998; Laurence et al. 2000), it is not known whether in vivo they may dimerize on the receptor. Certain chemokines such as RANTES/CCL5 are also known to form higher order aggregates, which have been shown to be important both in inflammation (Appay et al. 1999) and in inhibition of HIV infectivity (Wagner et al. 1998).

As mentioned above, chemokines are involved in both basal trafficking and inflammatory cell recruitment. Figure 1 highlights an interesting contrast between the receptors involved in basal trafficking (controlled by homeostatic chemokines), which tend to be specific, and those involved in inflammatory cell trafficking, which are shared by several chemokines. As is the rule for all such rules, this division is not strict, since CCR10 is considered to be constitutive, but is involved in certain skin inflammatory syndromes (Reiss et al. 2001;

Homey et al. 2002) while its ligand CTACK/CCL27 is upregulated by pro-inflammatory cytokines (Homey et al. 2002). The role of the homeostatic chemokines is to maintain the "normal" physiologic trafficking of cells for routine immunosurveillance requiring antigen sampling in the secondary lymphoid organs. Mice in which their genes have been deleted have demonstrated the importance of these receptors in development. Knockout mice deficient in either the CXCR4 receptor (Zou et al. 1998a,b; Ma et al. 1998; Tachibana et al. 1998) or SDF-1 (Nagasawa et al. 1996) die perinatally and the embryos show conspicuous defects in the hematopoietic and nervous systems. CCR7 and CXCR5 play important roles in homing of T cells and B cells respectively to secondary lymphoid tissue such as lymph nodes, and spleen, and both $CCR7^{-/-}$ and $CXCR5^{-/-}$ mice show severe defects in secondary lymphoid tissue architecture (Forster et al. 1996, 1999).

In inflammatory situations, the expression of inducible or inflammatory chemokines is upregulated. Control of expression of these chemokines is under the temporal control of pro-inflammatory cytokines. Pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ alone or in combination induce chemokine expression at sites of inflammation in nonlymphoid tissue (Segerer et al. 2000). In fact, the 3 ligands for CXCR3 were all identified upon upregulation with IFN-γ, hence their common names: IP-10, interferon-inducible protein 10 (Luster et al. 1988); MIG, monokine induced by interferon-γ (Farber 1990; Cole et al. 1998) and I-TAC/CXCL-5, interferon-inducible T-cell alpha chemoattractant (Rani et al. 1996). Activated cells, which possess appropriate chemokine receptors, upregulated during effector cell generation (Moser and Loetscher 2001) are then attracted to the site of inflammation. This is in contrast with resting peripheral cells, which express only homeostatic chemokine receptors. Different cell populations characterize different inflammatory responses, and the predominance of one cell type over another at a site of inflammation is probably dictated by the chemokines expressed at that particular site of inflammation/injury and the receptor expression pattern on the leukocytes.

The fact that chemokines interact with 7TM receptors not only differentiates them from other cytokines, but also renders their receptors tractable targets for therapeutic intervention. Screening for small-molecule inhibitors of cytokine receptors has been rather unsuccessful, whereas a huge proportion of drugs currently on the market act on members of the 7TM superfamily. There is, however, a feature of the chemokine family which may detract from their suitability as therapeutic targets, namely, the redundancy shown in Fig. 1, since multiple chemokines can bind certain receptors, and several chemokines can bind to more than one receptor. However, the levels of control in vivo such as temporal and spatial expression patterns, differential glycosaminoglycan (GAG) binding, differential receptor trafficking patterns as well as the different signaling pathways are far from fully delineated.

The interaction of chemokines on target cells is mediated through seven-transmembrane G protein-coupled receptors, which are usually of the Gi- Gi/Go type (Murphy 1996). Following activation, which has been reported to result in

dimerization (Mellado et al. 2001), chemokine receptors become either partially or totally desensitized to repeated stimulation—this process may be important in helping the cell sense a chemotactic gradient, so that it may move through the stroma to the inflammatory site. Binding of a chemokine to its receptor induces a conformational change in the cytoplasmic tail, which promotes the activation of the signaling cascades. Several intracellular signaling pathways, which are believed to influence each other through crosstalk, are induced and lead to cellular adhesion, migration, degranulation, and gene expression (Jiang et al. 1997; Szabo et al. 1998; Katanaev 2001). Some main components of the signaling events include release of intracellular Ca^{2+} via the phospholipase C (PLCβ) pathway and the activation of several isoforms of the protein kinase (PKC) family and phosphoinositide 3-kinase (PI3Kγ). One early event stimulated by ligand binding is the recruitment and activation of a heterotrimeric G protein complex leading to dissociation of the Gα subunit from the membrane-anchored G$\beta\gamma$-heterodimer (Gether and Kobilka 1998). The G$\beta\gamma$ subunit released transduces signaling events that lead to chemotaxis in motile cells in a Gα-independent manner (Parent and Devreotes 1999). Another G$\beta\gamma$-activated pathway involves PI-3 kinase γ (PI3Kγ) where PI3Kγ activation induces the production of phosphoinositil triphosphate (PIP3) (Jiang et al. 1997) and is involved in mitogen-activated protein kinase (MAPK) activation (Bondeva et al. 1998). Both effects are implicated in the chemotactic response. However, other factors mediated through small guanosine triphosphate (GTP)-binding proteins of the Rho-family, whose linkage to the G protein-coupled receptors is not fully understood, have been proved to be involved in chemotaxis (Hart et al. 1998). Chemokines also activate another set of cytoplasmic protein kinases, Janus kinases (JAKs), which phosphorylate signal transducers and activators of transcription (STATs) proteins, which affect gene expression (Mellado et al. 1998; Wong and Fish 1998). While the link between chemotaxis and PI3Kγ has been clearly established (Hirsch et al. 2000), the biological responses mediated by the JAK/STAT signaling pathways remain to be elucidated. As more insight is gained into this complex network, potential targets for therapeutic intervention should be discovered as has been postulated for an intracellular CCR2 receptor antagonist (Yokochi et al. 2001).

2 Cellular Recruitment and Chemotaxis Assays

In order for circulating leukocytes to reach sites of inflammation, they must cross the endothelial cell barrier. Leukocyte transmigration usually occurs at post-capillary venules, and is a multistep process first described by Butcher (1991) in which chemokines play at least two major roles, as illustrated in Fig. 3. Leukocytes first slow down by a selectin-mediated rolling, enabling the initial encounter with chemokines, which are presented on the endothelial cell surface by their immobilization on proteoglycans—introducing the second important intramolecular interaction in chemokine biology their interaction with GAGs.

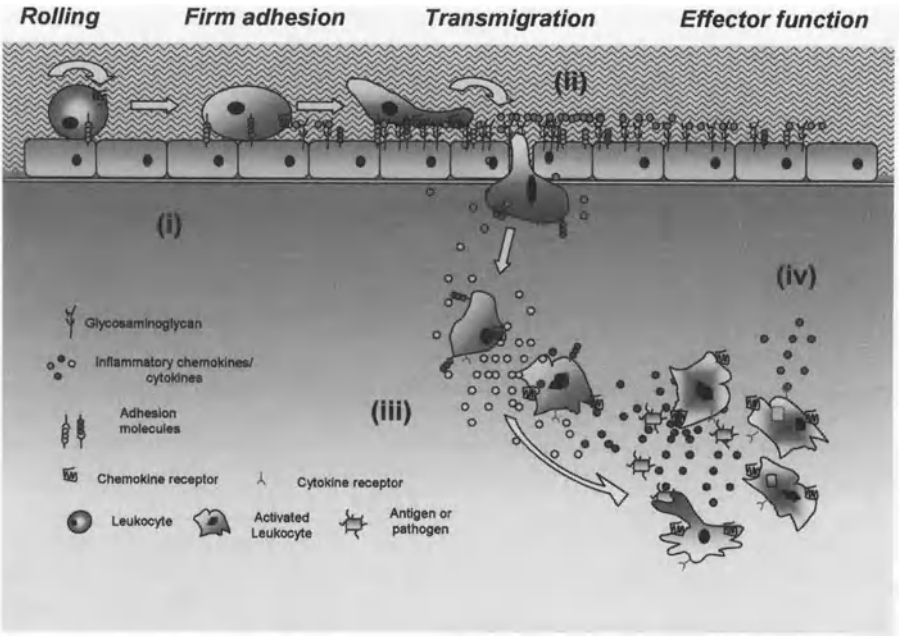

Fig. 3 Orchestrated cellular recruitment mediated by chemokines. Leukocytes circulating in the bloodstream, enter the underlying tissue through the endothelial cell layer (*i*), a process called transendothelial migration. (*ii*) The leukocyte is first slowed down by interactions between selectin/mucin molecules, which cause it to roll along the endothelium. Close to sites of inflammation, the endothelial layer is activated by pro-inflammatory cytokines, resulting in the upregulation of expression of adhesion molecules. Chemokines are secreted and are immobilized on the surface by binding to glycosaminoglycans (GAGs). The interaction of leukocytes with these immobilized chemokines changes the affinity of surface integrins, resulting in firm arrest of the leukocyte, followed by transmigration through the endothelium. (*iii*) Once the migrating cell has crossed the endothelial layer, it follows the haptotactic gradient of chemokines immobilized on the stroma and matrix of interstitial cells. (*iv*) When the cell reaches the site of inflammation, pro-inflammatory cytokines and chemokines induce distinct effector functions

Chemokine/receptor binding on leukocytes causes integrin activation resulting in increased avidity, and thus firm adhesion with adhesion molecules on the endothelial cell surface (Springer 1990; Dustin and Springer 1991). Once firm adhesion to the endothelial surface has been established, the leukocyte transmigrates from the lumen to the site of inflammation in the tissue by a process of haptotaxis (movement along a solid phase gradient). Chemokines bind GAGs, which are present in the extracellular matrix, to create such a gradient or a "guideline" that the cells may use to navigate towards the site of inflammation, where they exert their effects. It is probable that, in vivo, several gradients of different chemokines are present in the tissue, separated temporally and spatially [reviewed in Moser and Loetscher (2001) and Devalaraja and Richmond (1999)]. Specificity introduced to the system both spatially and temporally has been demonstrated by Foxman et al. (1997). In this elegant study, the authors

demonstrate that neutrophils navigate towards agonists in a spatially defined and sequential manner.

Recently, the first example of reverse cell migration induced by a chemokine has been reported (Poznansky et al. 2000). This follows the observation that while there are high levels of SDF-1 (a chemoattractant for mature T lymphocytes) in bone marrow and thymic tissue, there is a paucity of this cell type in these areas. This may be explained by a theory put forward by Zlatopolskiy and Laurence (2001) who propose that differential signaling mechanisms control forward or reverse cell migration in response to a chemokine, depending on the critical concentration of that chemokine.

The ability of chemokines to recruit cells has been mimicked in vitro by chemotaxis assays. The classical in vitro assay of the chemotactic function of chemokines has been a system using the Boyden chamber or a similar method of cell chemotaxis [methods reviewed in Wilkinson (1998)]. Briefly, these experiments involve the monitoring of cell migration across a permeable membrane in the positive direction of the agent that is being tested. In these experiments, the chemotactic gradient is created artificially by increasing the chemokine concentration in the lower wells. While this system is widely used as a primary test for the chemotactic ability of a molecule, as with all in vitro methods, it is only an imitation of the in vivo situation. More sophisticated chemotaxis assays have also been described whereby the cell must migrate across an endothelial cell layer and, to make the system more physiological, a shear flow can be applied (Cinamon et al. 2001). However, the best demonstration of the chemoattractant properties should be those in vivo.

Experiments using a flow chamber and endothelial cells to study the differential roles of chemokine in monocyte arrest and extravasation have shown that while GRO-α/CXCR2 is important in transforming monocytes from a rolling state to one of firm arrest, MCP-1/CCL2 is involved in the subsequent shape change and transmigration step (Weber et al. 1999). It is noteworthy that in these studies the effects of MCP-1/CCL2 could only be seen under flow conditions, suggesting that flow may play an important role in the transmigration step, an observation which was also made by another group (Cinamon et al. 2001).

While it would appear simple to monitor cellular recruitment by injecting the chemokine of interest into an animal, in vivo chemotaxis has not been widely used. However, certain methods have been reported where the chemokine is administered into a "pocket" in an animal. The pocket may be a naturally occurring space in the animal; for example, the chemokine can be applied to the lung via the intra tracheal route (Hisada et al. 1999), or into the peritoneal cavity by injection (Z. Johnson and A.E.I. Proudfoot, unpublished observations) or implantation of a chemokine-soaked sponge (Fine et al. 2000). In other situations the pocket may be artificial—for example, by creating an air pouch on the animal's back (Takano et al. 1999). Cellular recruitment can also be induced by the administration of a chemokine by intra dermal injection and MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7 in rabbits have all been shown to recruit mono-

cytes to the site of injection in the skin (Van Damme et al. 1992). Eotaxin, which was identified by biochemical means as the eosinophil recruitment factor from the BAL of sensitized guinea pigs, when injected into guinea pig skin induces an accumulation of eosinophils as would be expected (Jose et al. 1994). Interestingly, however, RANTES/CCL5, which is also an eosinophil chemoattractant in vitro, will only attract an inflammatory cell infiltrate of eosinophils and monocytes following injection into the skin of dogs previously subjected to helminth infection (Meurer et al. 1993). The ability of RANTES/CCL5 to recruit eosinophils only in sensitized subjects was further demonstrated in man where RANTES/CCL5 injection into skin of non-atopic subjects did not elicit an inflammatory response, compared with the predominantly eosinophilic infiltrate observed following RANTES/CCL5 injection in atopic patients (Beck et al. 1997). Intradermal injection of the prototypic CXC chemokine IL-8/CXCL8 in rabbits induces plasma exudation which is dependent on specific recruitment of neutrophils (Colditz et al. 1989; Foster et al. 1989; Rampart et al. 1989; Colditz et al. 1990), and the same exclusive recruitment of neutrophils induced by IL-8/CXCL8 has been confirmed in man (Leonard et al. 1991; Swensson et al. 1991).

In addition to the injection of specific chemokines in vivo, chemotactic agents such as thioglycollate or the bacterial cell wall component lipopolysaccharide (LPS) have been shown to produce a pronounced and specific cellular infiltrate following injection in vivo. LPS induces a rapid initial recruitment of neutrophils followed later by monocytes into the host tissue (Evans et al. 1989; Ghosh et al. 1993), which has been shown to be partially mediated by localized production of chemokines, including MCP-1/CCL2, RANTES/CCL5, and MIP-1α/CCL3 and -β/CCL4 (Kopydlowski et al. 1999). Injection of aged thioglycollate medium has been used as a method for eliciting peritoneal neutrophils or macrophages for many years, with neutrophil recruitment occurring at 4 h (Rodrick et al. 1982) while 3–4 days after injection the predominant cell type in the peritoneal cavity are macrophages (Mishell 1980). Macrophage recruitment after thioglycollate administration involves MCP-1/CCR2, since both the MCP-$1^{-/-}$ and the $CCR2^{-/-}$ mice show impaired responses in this model (Boring et al. 1997; Kurihara et al. 1997; Kuziel et al. 1997), and administration of an anti-CCR2 antibody also significantly inhibits cell recruitment in this model (Mack et al. 2001).

3
Macrophage Chemokine and Receptor Expression Involved in Disease

The aim of this chapter is to concentrate on the effects of chemokines on the monocyte/macrophage leukocyte phenotype in disease, which is the central theme of this book. Several chemokine receptors are expressed on monocytes which are responsible for the rapid and directed migration of these cells from the circulation into tissue, where they mature into phagocytic macrophages involved in cell-mediated host defense against infection. It is very important to note that macrophages have a different chemokine-receptor expression pattern

from circulating monocytes (Kaufmann et al. 2001). A good example of differential expression is shown by the RANTES receptors. Circulating monocytes express high levels of CCR1, but very low levels of CCR5 and CCR3. Thus RANTES/CCL5 induces a robust response of monocyte chemotaxis, while eotaxin/CCL11 and MIP-1β/CCL4, specific ligands for CCR3 and CCR5 respectively, induce only moderate responses (Proudfoot et al. 1999) Furthermore, the amino terminally modified variant AOP-RANTES, which is a potent agonist of CCR5, but not of CCR1 (Proudfoot et al. 1999) is not able to induce monocyte chemotaxis (Simmons et al. 1997). On the contrary, AOP-RANTES induces a robust calcium response on monocytes that have acquired the macrophage phenotype in vitro, while it is not able to elicit this response in freshly isolated monocytes (Proudfoot et al. 1999), indicative of the altered expression pattern of chemokine receptors on monocytes and macrophages.

The levels of chemokines and their receptors in macrophage-mediated pathologies such as rheumatoid arthritis (RA); multiple sclerosis (MS) and arteriosclerosis have been extensively studied. In RA, macrophages are believed to play a pivotal role in nefarious activities such as joint destruction, and the synovial fluid from RA patients has been shown to contain a variety of chemokines which attract monocytes, including macrophage inflammatory protein (MIP)-1α/CCL3 (Hosaka et al. 1994; Koch et al. 1994), MIP-1β/CCL4 (Koch et al. 1995), MCP-1/CCL2 (Koch et al. 1992; Villiger et al. 1992) and RANTES/CCL5 (Rathanaswami et al. 1993; Volin et al. 1998). A study of the chemokine receptors expressed in the three leukocyte-trafficking compartments of RA (peripheral blood, synovial fluid and synovial tissue) showed that CCR1 and CCR2 are highly expressed on normal and RA peripheral blood monocytes, but are expressed at low levels on these cells in the synovial fluid, suggesting that they are involved in the recruitment process. Other receptors such as CCR3, CCR4, and CCR5 were found to be upregulated on peripheral blood mononuclear cells (PBMC) from arthritic patients compared to normal samples (Katschke et al. 2001). These results suggest that differential chemokine-receptor expression patterns play important roles in monocyte/macrophage recruitment and retention in disease.

In another chronic autoimmune disease in which macrophages are believed to play a major role, MS, the chemokines IP-10/CXCL10, Mig/CXCL9, which act via CXCR3, and RANTES/CCL5, which acts via CCR1, CCR3 and CCR5 were all shown to be upregulated in the cerebrospinal fluid (CSF) of patients during an MS attack, whereas MCP-1/CCL2 levels, which acts via CCR2, were decreased (Sorensen et al. 1999). The study of the receptor levels in the CSF and on cells present in MS lesions revealed that CXCR3 is elevated in the CSF compared with the level of expression in the peripheral blood (Sorensen et al. 1999). Other studies have shown that CCR5 receptor expression is increased on T cells and macrophages both in the CSF and in MS lesions (Balashov et al. 1999; Sorensen et al. 1999; Strunk et al. 2000). Further evidence that CCR5 plays an important role in the progression and pathology of MS comes from the observation that MS patients that are heterozygous for the CCR5Δ32 allele, which codes a non-

functional CCR5, have prolonged disease-free intervals between MS attacks compared with individuals expressing wildtype CCR5 (Sellebjerg et al. 2000). Recently the same phenomenon of decreased CCR1 expression in recruited cells that was observed in the synovial fluid of RA patients was seen in MS lesions, whereas CCR5 expression is increased (Trebst and Ransohoff 2001).

The macrophage is known to play a major role in arteriosclerosis, and the observation of high levels of one of the major monocyte/macrophage chemoattractant, MCP-1/CCL2, in atherosclerotic plaques taken from patient samples is not surprising (Nelken et al. 1991). The role of this chemokine and its receptor CCR2 is well borne out by their deletion in mice as is described below. In the remainder of this chapter, we will describe the approaches that would interfere with the role of chemokines as potential strategies to interfere with the inflammatory process in disease, and review the current status of chemokine anti-inflammatory therapeutics.

4 Proof of Concept of Interference with the Chemokine System as Anti-inflammatory Therapies

Before describing the therapeutic strategies that could be applied in man, it is worthwhile to briefly review the evidence that has validated the role of the chemokine system and macrophage biology in animal models using genetic manipulation.

The first genetic manipulation approach is the creation of transgenic mice, an overexpression of a specific chemokine. The theoretical advantage of this approach is that high levels of the chemokine may be maintained at a specific site for a sustained period, perhaps mimicking the effects of an inflammatory response. Surprisingly, the targeted overexpression of MCP-1/CCL2 to the pancreas under the control of the insulin promoter showed that, while extensive monocytic infiltration was induced, this was not paralleled with inflammation, leading to the important observation that cellular recruitment alone is not sufficient to create an inflammatory response, but that a second activation or danger signal is required, as has been suggested by the work of Matzinger (1994). Similarly, overexpression of MCP-1/CCL2 in alveolar cells, so that the chemokine was secreted into the bronchoalveolar space, resulted in an increase in monocytes and lymphocytes in the bronchoalveolar lavage, again without an accompanying inflammation (Gunn et al. 1997)

The alternative genetic approach is to generate knockout mice by gene deletion. In contrast to deletion of constitutive receptors and ligands, which have shown striking phenotypes, inflammatory receptor, and ligand-knockout mice have no unusual phenotype under normal conditions, but do show phenotypes when subjected to inflammatory stress. A good example is the MIP-1α/CCL3 knockout, which only demonstrated a phenotype in infection with Coxsackie virus (Cook et al. 1995). Knockout mice in which the gene for MCP-1/CCL2 has been deleted have been far more informative. These mice showed a fourfold re-

duction in recruitment of macrophages in response to thioglycollate administered peritoneally, even though the exact mechanism of cellular recruitment remains to be elucidated (Gosling et al. 1999). Further evidence for the role of MCP-1/CCL2 in monocyte recruitment was shown in a delayed-type hypersensitivity (DTH) model. In the MCP-$1^{-/-}$ mouse, an impairment of macrophage accumulation in DTH lesions was observed compared with wildtype mice, despite the fact that the swelling response was similar in both wildtype and gene-deleted mice. Perhaps the most convincing data generated from these mice is in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE). In this study, MCP-$1^{-/-}$ mice were shown to be significantly resistant to EAE following active immunization, with a corresponding impairment of recruitment of macrophages to the CNS (Huang et al. 2001). These experiments demonstrated a very important fact with respect to redundancy. While MCP-1/CCL2 acts only on CCR2, it is not the only chemokine to activate this receptor; yet the deletion of this chemokine mirrored the effect of deleting the receptor itself. In other words, the other CCR2 ligands were not able to compensate. $CCR2^{-/-}$ mice have shown similar macrophage recruitment defects, where macrophage recruitment in response to peritoneal thioglycollate administration is severely impaired. In murine models of arteriosclerosis, both MCP-1/CCL2 and $CCR2^{-/-}$ mice have unequivocally demonstrated the importance of this receptor/ligand pair (Gosling et al. 1999)

Knockout mice do not always confirm the role for certain ligands or receptors, since deletion of MIP-1α/CCL3 and CCR5 showed that these mice remained fully susceptible to EAE (Tran et al. 2000). However, MIP−1α/CCL3 knockout mice show a decrease in cuprizone-induced demyelination (McMahon et al. 2001). These results are in contrast to the neutralization of MIP-1α as described below, and with the strikingly high levels of expression of CCR5 in MS lesions, we believe that interpretations with knockout mice should be taken with care, as compensatory mechanisms may occur in gene-deleted animals—especially in a system as "redundant" as the chemokine system.

5 Anti-inflammatory Strategies

There are several approaches that could be undertaken to inhibit the chemokine-mediated recruitment of monocyte/macrophages into inflammatory sites, which are summarized in Fig. 4. The most frequently applied strategy is that of preventing the interaction between the chemokine and its receptor, and to this end several approaches have been adopted.

The use of neutralizing monoclonal antibodies, principally against the chemokines themselves, has been used extensively in animal models of disease, although surprisingly, few are being developed for therapeutic use perhaps a reflection of the belief that orally available small molecule receptor inhibitors would supersede the use of antibodies. However, published results prove that the use of neutralizing antibodies against specific chemokines can successfully

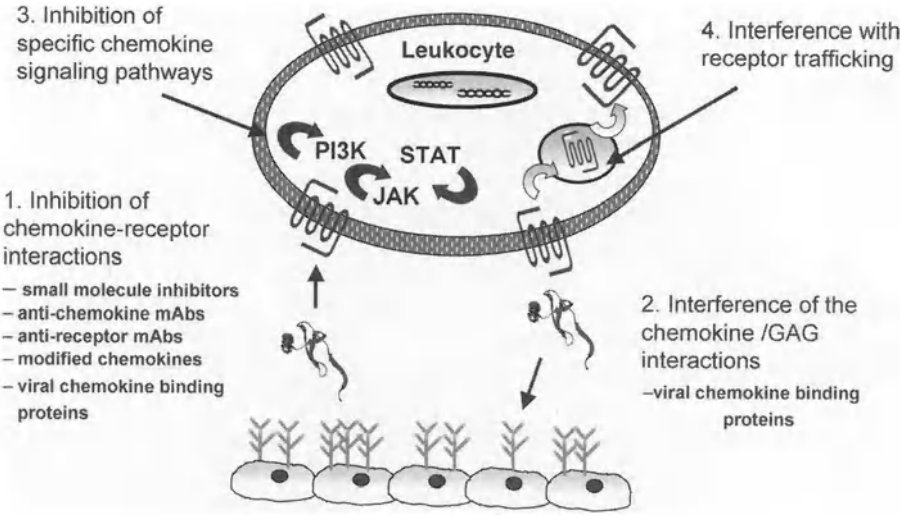

Fig. 4 The possibilities of intervention points in the chemokine system to prevent inflammation. Four possible strategies are depicted. (*1*) The most frequent strategy applied to date, which is the prevention of chemokine/receptor interactions. (*2*) Chemokines are believed to interact with cell surface glycosaminoglycans in order to form the chemotactic gradient. (*3*) Chemokines may have specific signalling pathways. (*4*) Receptor endocytotic pathways have been shown to differ for different chemokine receptors

block specific inflammation in animal models. The blockade of several different CC chemokines in T helper (Th)2 inflammation using a murine model of airways inflammation induced by ovalbumin sensitization revealed that there is a coordinated action of chemokines in the inflammatory process which orchestrates the complete response. Eotaxin/CCL2 and MCP-1/CCL2 were shown to be important for lung inflammation (eosinophil and monocyte infiltration respectively) and bronchial hyperreactivity (BHR), whereas neutralizing MIP-1α/CCL3 had little effect. Use of a neutralizing antibody against the CXC chemokine IL-8/CXCL8 was also shown to be effective at blocking reperfusion-associated lung injury in a rabbit model (Sekido et al. 1993).

Similar efficacious treatments using this approach have been demonstrated in Th1 disease models. In collagen-induced arthritis in rodents, treatment with an anti-MCP-1/CCL2 mAb improved disease by significantly reducing the number of infiltrating macrophages into the lesions, correlating with a reduction in ankle swelling by 30% (Ogata et al. 1997). A neutralizing anti-RANTES/CCL5 polyclonal antibody similarly improved adjuvant induced arthritis by significantly reducing the cellular infiltrate, maintaining joint integrity and preventing bone destruction (Barnes et al. 1998). In EAE, macrophage accumulation has been shown to correspond with levels of MCP-1/CCL2 at the site of inflammation (Ransohoff and Tani 1998). Administration of an anti-MCP-1/CCL2 antibody prevented relapses in rodent EAE (Karpus and Kennedy 1997), suggesting

that the macrophage plays a role in this process. On the other hand, anti-MIP-1α/CCL3 treatment prevented the disease onset in an acute model, but not in relapsing and remitting EAE (Karpus and Kennedy 1997). A mAb against rat MCP-1/CCL2 was also used to demonstrate that MCP-1 is responsible for infiltration of monocytes in bleomycin-induced lung injury in rats (Sakanashi et al. 1994). An antibody to MCP-1/CCL2 was able to inhibit monocyte migration across an in vitro model of the blood brain barrier (BBB) by 85%, which supports the role of MCP-1/CCL2 and its receptor CCR2 in recruiting monocytes into the brain (Weiss et al. 1998).

Neutralizing antibodies to rodent chemokine receptors are not widely available, introducing a limiting factor to correlate data obtained in rodent models to receptor antagonism in human disease. Recently, mAbs against murine CCR2 and CCR5 have been described (Mack et al. 2001), which hopefully will provide more information as to the efficacy of blocking these receptors in macrophage-mediated inflammation. While Eotaxin/CCL11-mediated eosinophil recruitment in the guinea pig has been shown to be blocked by a mAb against guinea pig CCR3 (Sabroe et al. 1998), there is no report of its efficacy in airways inflammation.

A second approach to preventing ligand-mediated receptor activation is to use a modified ligand that retains high-affinity binding to the receptor but has lost the ability to induce signaling, and is thus a receptor antagonist. The amino terminal region has been shown by many studies to be crucial for receptor activation, and therefore such receptor antagonists have been generated by the modification of this region, either by truncation or elongation. There are several examples described in vitro of the former approach with IL-8, (Clark-Lewis et al. 1991), RANTES/CCL5 (Gong et al. 1996) and MCP-1/CCL2 (Zhang and Rollins 1995; Kim et al. 1996), but not many in vivo. However, the truncated MCP-1/CCL2 analog (9–76)-MCP-1/CCL2 was shown to have very good efficacy in the murine model of arthritis in MRL-*lpr* mice; its therapeutic administration reduced disease symptoms (Gong and Clark-Lewis 1995). The elongation approach has been extensively studied in vivo with the RANTES analog, Met-RANTES (Proudfoot et al. 1996), where it has shown efficacy in a variety of models which implicate monocyte/involvement such as collagen-induced arthritis (Plater-Zyberk et al. 1997), glomerular crescentic nephritis (Lloyd et al. 1997), organ transplant (Grone et al. 1999), and colitis (Ajuebor et al. 2001).

While the power of biological therapeutics should not be ignored, the dogma of the pharmaceutical industry is based on the superiority of orally bioavailable small molecule inhibitors. Chemokine receptors, which belong to the GPCR class, are ideal targets. However, the majority of these receptors have small ligands, and while chemokines are small proteins, they are significantly larger compared to ligands such as histamine, adrenaline, or dopamine. Moreover, the fact that they bind several ligands, and that ligand binding can be allotropic because the ligands do not necessarily bind to the same site (Cox et al. 2001), renders the search for small molecules more challenging. However, the combination of focused high-throughput screening and medicinal chemistry has led to

the development of highly-potent small molecule chemokine receptor antagonists, for which the number in the patent literature is now over 200—no mean feat since this receptor class was only identified just over a decade ago. Certain small molecule antagonists lack receptor selectivity, which may in fact be advantageous, as has been suggested for the dual CCR1/CCR3 inhibitor (Sabroe et al. 2000). However, specific small molecules have been described in the peer-reviewed literature, such as the CCR1 inhibitor BX471, CXCR2 inhibitors, and the CCR5 inhibitors, TAK779 and SCH-C, although TAK779 also has significant inhibitory properties for the closely related receptor, CCR2. To date the only anti-inflammatory in vivo data published are for the CCR1 antagonist BX471, where the compound has been shown to be efficacious in rat heart transplant rejection by reducing RANTES-mediated monocyte adhesion on inflamed endothelium (Horuk et al. 2001), in renal fibrosis by reducing macrophage and lymphocyte infiltration into the kidney (Anders et al. 2002), and in a rat model of EAE (Liang et al. 2000). The CCR5 compound TAK779 demonstrates an important feature in that it is a non-competitive inhibitor, since it binds to a cavity in the transmembrane domains rather than to the ligand-binding site(s) (Dragic et al. 2000). This is particularly important in view of the fact that certain receptors such as CXCR3 have distinct ligand-binding sites (Cox et al. 2001), which makes the identification of competitive inhibitors impossible.

It should be noted that while the published data on small molecule inhibitors are still very limited, since clinical trials have started for some of these inhibitors, numerous conference presentations indicate that there is considerable progress.

6
The Role of the Macrophage in Infection: HIV

In this chapter we have focused on the role of chemokines and their receptors in inflammation, but the importance of these proteins and the macrophage with respect to HIV cannot be ignored. It is widely believed that the macrophage is the cell involved in viral transmission, although primary transmission through infection of T lymphocytes and the dendritic cell cannot be excluded. For many years it was known that the high-affinity interaction with CD4 (Dalgleish et al. 1984) was not sufficient for infection of host cells, but it was only in 1996 that chemokine co-receptors were identified as the essential co-receptors (reviewed in Berger 1997). The initial binding of HIV to CD4 on the target cell surface leads to a conformational change in gp120, a viral envelope surface glycoprotein, which then interacts with the chemokine receptor, inducing a second conformational change which exposes another viral protein gp41, also known as the fusion peptide, that is inserted into the host membrane, resulting in membrane fusion and virus entry into the cell. It had been shown a few months before the discovery of the chemokine receptors as co-receptors that three chemokines, RANTES/CCL5, MIP-1α/CCL3, and MIP-1β/CCL4 were able to reduce HIV infection of cells (Cocchi et al. 1995). This immediately indicated that a

blockade of the chemokine receptors could be an anti-HIV therapeutic strategy. This strategy was borne out by the finding that homozygous individuals with a mutation in the CCR5 gene (Δ32-CCR5), which prevents the cell surface expression of the receptor, are resistant to infection by HIV (Samson et al. 1996). It is still not completely clear why transmission is almost exclusively mediated by CCR5-using HIV-1 strains, also called R5 strains, and not by CXCR4 or X4 strains, since macrophages express all the required components, CD4, CCR5, as well as CXCR4.

The establishment of the principle that infection could be prevented with chemokine receptor antagonists caused a huge effort by the pharmaceutical industry to find small-molecule inhibitors. CCR5 has proved more tractable than CXCR4, with nanomolar compounds described in the literature (Baba et al. 1999; Strizki et al. 2001). SCH-C has the advantage of having very good oral bioavailability, allowing an in vivo proof of concept in severe combined immunodeficiency (SCID) mice, since studies in non-human primates were excluded, owing to the high degree of specificity of the compound for the human receptor (Strizki et al. 2001). The start of clinical trials with this molecule has been announced at conferences. Many more chemical series have been granted patent filing, with the total number now being over 50. On the other hand, only one CXCR4 inhibitor series has been described in the literature (Donzella et al. 1998), which has the handicap of not being very "drug-like," with a molecular weight over 1,000 Da.

Studies with modified chemokines as potential anti-HIV therapies have provided very interesting information as to the biology behind HIV inhibition, but their clinical development programs are not yet known. Chemokines could inhibit HIV infection by two mechanisms. First, steric hindrance through acting as pure competitive or non-competitive inhibitors as is the case for TAK779, or alternatively by inducing endocytosis of the receptor, thereby stripping the essential co-receptor from the cell surface. While the first mechanism obviously plays an important role, the efficacy of the second mechanism was demonstrated by a chemically modified RANTES/CCL5 variant, created by the chemical coupling of a pentacarbon alkyl chain to the N-terminus of the chemokine, and hence named aminooxy pentane RANTES (AOP-RANTES) (Simmons et al. 1997). This analog was shown to be far more potent than the truncated RANTES receptor antagonist (9–68)-RANTES (Arenzana-Seisdedos et al. 1996) and Met-RANTES (Simmons et al. 1997) and was subsequently shown not to be a CCR5 antagonist but rather a superagonist of this receptor in that it caused enhanced phosphorylation of CCR5 (Oppermann et al. 1999) as well as enhanced receptor downmodulation properties (Mack et al. 1998). But more surprising was its property to prevent recycling of functional receptors (Mack et al. 1998). Although the mechanism of this phenomenon is not yet fully elucidated, it appears that while it does allow receptor recycling, the receptor is immediately re-internalized (Signoret et al. 2000), thereby rendering it inaccessible to the HIV gp120 interaction. The efficacy of preventing cell-surface receptor expression has been elegantly demonstrated using another approach by the genetic addition of the

endoplasmic retrieval peptide KDEL to RANTES/CCL5, MIP-1αCCL3, or SDF-1/CXCL5 (Chen et al. 1998) to create so-called intrakines, which bind to newly synthesized receptors CCR5 or CXCR4, respectively, and inhibit their transportation to the cell surface.

7 Can We Learn Strategic Therapeutic Approaches from Pathogens?

Pathogens have evolved over the millennia in an attempt to evade the host's immune response. Examples of "silent" (non-viral) pathogens are leeches and ticks that are able to feed on their host in the absence of an inflammatory or allergic response. Anti-cytokine and chemokine activities have been identified in tick saliva (Gillespie et al. 2001; Hajnicka et al. 2001) although the molecular identities of these molecules are as yet unknown. However, the best documented pathogens are viruses whose rapid mutational capacities could allow them to become immunologically silent or to devise other methods to escape the host response. Several mammalian viral species have been found to express arrays of immunomodulating molecules, such as chemokines, chemokine receptors, or chemokine-binding proteins, which are used to increase survival and dissemination in vivo and/or decrease the recognition of the virus by its host. Virally expressed chemokines may enhance dissemination by either inducing the recruitment of host cells (McFadden and Murphy 2000) or inducing a Th2-shift of the host immune response (Weber et al. 2001). Human herpes virus-8 encoded viral MIP-II binds potently to both CC and CXC receptors and can efficiently block HIV infection (Zhou et al. 2001). In fact, this molecule crosses one of the barriers of chemokine receptor specificity in that it is able to inhibit both R5 and X4 HIV viral infection (Kledal et al. 1997), a feat that man has yet to achieve. In a rat model for glomerular nephritis, the in vivo administration of vMIP-II attenuates the disease (Chen et al. 1998) and studies on ischemic brain injury have shown that it might also be useful in the therapeutic intervention of stroke (Takami et al. 2001). A soluble chemokine receptor antagonist, MC148R, is expressed by the molluscum contagiosum virus that might become useful as an anti-inflammatory molecule, as it has been shown to be highly specific for CCR8 and hence for inhibiting monocyte invasion and dendritic cell function (Luttichau et al. 2000).

Poxviruses have revealed another interesting class of chemokine inhibitors in the guise of molecules that bind and neutralize chemokines, but which have no mammalian homologs. These binding proteins fall into different classes (Lusso 2000). The first, belonging to the T1 or p35 type (Lalani and McFadden 1997; Stine et al. 1999) show promiscuous binding to several beta-chemokines and are able to inhibit in vitro chemokine activities, thereby interfering with chemokine receptor interactions (Lalani and McFadden 1997; Alcami et al. 1998). Another chemokine-binding protein, M3, encoded by the murine γ-herpesvirus-68, binds members of all four classes of chemokines (Bridgeman et al. 2001). Its expression prevents the recruitment of T cells, but not of B cells and neutrophils

(Parry et al. 2000), which seems to have an evolutionary advantage as the virus replicates in B cells. Another class is represented by the myxoma viral protein, M-T7, which is unable to inhibit in vitro activities despite the fact that it interacts in vitro with a broad range of CC and CXC chemokines. Its mode of action is believed to be through interaction with their heparin-binding domains (Lalani et al. 1997), thereby inhibiting the formation of a chemokine gradient on the endothelial surface and adds weight to the approach suggested in Fig. 3 as a possible anti-inflammatory strategy. The administration of this protein in animal models of inflammation has validated this concept (Liu et al. 2000). Lastly, functional chemokine receptor-like molecules are expressed by some viruses. The receptor US-28 is expressed by the human cytomegalovirus, presumably to help its dissemination in the human host by mediating migration of infected smooth-muscle cells, a fact that can be linked to the acceleration of vascular disease (Streblow et al. 1999, 2001). It is certain that with more structural information and biological data becoming available, viruses could perhaps teach us how to create molecules with, for example, the property described above of vMIP-II, the only molecule that can block both R5 and X4 HIV infection.

8 Conclusions

There is sufficient evidence, some of it summarized in this chapter, that despite the redundancy apparent in the chemokine system, that interference with the system has a significant therapeutic potential. The therapeutic areas encompass those involving macrophage function, including many inflammatory pathologies, as well as infectious disease such as AIDS, where the macrophage is believed to play a pivotal role. It is, perhaps, in the latter area where chemokine antagonists are most advanced, where small-molecule inhibitors of the principal co-receptor, CCR5, are already in clinical trials.

We have described some of the many results obtained with an approach used by that of the biotechnology field: protein therapeutics. Both neutralizing antibodies and modified chemokines in the guise of receptor antagonists have shown to have efficacy. While it is well accepted that an orally available small-molecule inhibitor is the preferred molecule of choice, antibody therapies have a well-proven track record in the clinic. Hopefully biological therapeutics will find their niche as anti-chemokine strategies, in the form of antibodies, receptor antagonists, or pathogen-derived chemokine-binding proteins.

To date the focus has been on receptor antagonists, but as we have illustrated in Fig. 3, we believe that there are many other approaches, some of which are not even depicted in this illustration. The signaling pathway are far from being fully delineated, and may provide attractive targets which interfere not only with chemotaxis, but with other aspects of chemokine biology such as cellular activation, differentiation, angiogenesis, and metastasis, to name but a few. The approaches to block chemokine action could also be more indirect, such as heterologous receptor desensitization, interference with chemokine gene transduc-

tion by gene therapy, or prevention of receptor expression for the prevention of HIV infection using subtle strategies such as trapping in the endoplasmic reticulum. In summary, the relatively new area of immunology opened up by the field of chemokine biology promises many novel therapeutic approaches.

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Antimicrobial Peptides

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Abstract Antimicrobial peptides, mainly defensins and cathelicidins, are abundant components of granulocytes, Paneth cells of the small intestine, inflamed epithelia, and rabbit alveolar macrophages. There is increasing evidence that in these settings antimicrobial peptides and larger proteins contribute to microbicidal activity and other host defense functions. However, antimicrobial peptides and antimicrobial proteins (with the exception of lysozyme) are present at most in small amounts in most types of macrophages. In some cases, antimicrobial peptides may be difficult to detect at the protein level because macrophages lack granules, the large preformed storage compartment for antimicrobial peptides of granulocytes and Paneth cells, and may instead synthesize antimicrobial substances continually or on demand. Alternatively, histones, other nucleoproteins, or as yet unrecognized polypeptides or nonprotein components may contribute to oxygen-independent killing in macrophages.

Keywords Cathelicidins, Defensins, Histones, Host defense

1 Overview

Antimicrobial peptides are polypeptides of mass less than 10 kDa. They are found in a host-defense context and manifest antimicrobial activity in vitro when tested under conditions found in their proposed sites of action. Antimicrobial peptides may act alone or in concert with other host-derived molecules. In the last 20 years, hundreds of different antimicrobial peptides have been discovered in the phagocytes, epithelia, and secretions of vertebrates, and in the blood cells and tissues of invertebrates. The peptides comprise one end of a continuum of gene-encoded antimicrobial molecules that range in size from the large protein complexes that form complement pores, through intermediate size (10–100 kDa) proteins that can target and disrupt microbial membranes or sequester essential micronutrients.

It is important to acknowledge "up front" that most available information about antimicrobial peptides comes *not* from studies of macrophages, but from other cells—mainly polymorphonuclear leukocytes (PMN) and epithelial cells. Epithelia, most prominently the epidermis, synthesize antimicrobial peptides constitutively or in response to signals associated with injury, inflammation, or infection. In the PMN of many species, antimicrobial peptides are synthesized constitutively and in such abundance that they constitute the predominant polypeptides in crude cell extracts. Polymorphonuclear leukocytes store their antimicrobial peptides in cytoplasmic granules of several types. Some of these subcellular organelles (e.g., the primary or azurophil granules of human PMN) preferentially deliver their contents to phagosomes, while others (e.g., the secondary or specific granules of human PMN) release their contents mostly to the cell surface and external milieu. In the inflamed epidermis, the site of antimicrobial peptide deposition is not yet well characterized. In either case, the presence of high concentrations of stored antimicrobial peptides has facilitated their biochemical detection, recovery, and analysis. In contrast, with the prominent exception of rabbit alveolar macrophages, the macrophages examined to date have not contained high concentrations of antimicrobial peptides other than histones or fragments thereof.

Nevertheless, even macrophages from mice, an animal whose PMN are relatively deficient in antimicrobial peptides (Eisenhauer and Lehrer 1992) clearly possess antimicrobial mechanisms other than the production of reactive oxygen or nitrogen products (Shiloh et al. 1999). Several possible scenarios have been considered. First, there may a substantial contribution to antimicrobial activity from phagosome acidification, nutrient depletion (e.g., depletion of iron or tryptophan), or other mechanisms not dependent on the delivery of high concentrations of antimicrobial substances into the phagosome. Second, sufficient amounts of antimicrobial substances may be delivered to the phagosome by active synthesis coupled with vesicular transport, even in the absence of a storage compartment. Lysozyme (muramidase), a peptidoglycan-degrading enzyme found in most macrophages, is more often secreted than retained by murine

peritoneal macrophages. Third, an external source of antimicrobial substances, including granule proteins and peptides from PMN or histones from phagocytized and degraded nuclear material could contribute to killing in vivo. Finally, as yet unrecognized antimicrobial substances, including autologous histones and histone fragments may contribute to phagocytic killing in macrophages. We will first discuss the biology of antimicrobial peptides in mammalian polymorphonuclear leukocytes before reviewing the more limited information available in the diverse types of animal macrophages.

2 Antimicrobial Peptides in PMN

Within each PMN there are several thousand membrane-bounded cytoplasmic structures—"granules"—whose contents include cationic polypeptides complexed to an anionic proteoglycan matrix (Olsson 1969; Parmley et al. 1986). The granules are selectively recovered from broken PMN by differential centrifugation, and their contents can be extracted by acidic solvents. The extract typically consists of four groups of proteins (Modrzakowski et al. 1979; Greenwald and Ganz 1987) that can be separated by molecular weight. In the highest molecular weight fraction (>50 kDa), myeloperoxidase and lactoferrin are the predominant proteins, accompanied in some species by the somewhat less abundant B/PI (bactericidal/permeability inducing protein). In human PMN, the next fraction consists of ~30-kDa serine proteases (elastase, cathepsin G, proteinase 3) accompanied by azurocidin/CAP37, their enzymatically inactive homolog. The third fraction (~14 kDa) is predominantly lysozyme, but also contains the pro-forms of antimicrobial cathelicidin peptides. The last fraction (3–4 kDa), which is especially abundant in human and rabbit PMNs, contains defensins.

From the cellular content of the various antimicrobial polypeptides and the volume occupied by their cytoplasmic granules, it can be estimated that each of the major peptides and proteins exists at multi-milligram/ml concentrations within these organelles. Electron micrographs of phagocytic PMN suggest that the granule contents are deposited onto the surfaces of ingested microbes with relatively little dilution.

3 Defensins

Mammalian defensins (Ganz et al. 1985a; Selsted et al. 1985) are a family of genetically related peptides that possess a framework of six cysteines with three characteristic disulfide linkages. Based on the spacing of the cysteines and the pattern of their connections, defensins are further subdivided into α-, β-, and θ-defensins. All three families have been found in PMN. α-Defensins occur in PMNs from humans and other primates, rats, hamsters, guinea pigs, and rabbits. β-Defensins were found in cattle (Selsted et al. 1996) and fowl (Harwig et

al. 1994), and θ-defensins were identified in rhesus monkeys (Tang et al. 1999). The PMN of mice, pigs, sheep, and horses apparently lack defensins altogether. In Paneth cells, a specialized granule-rich epithelial cell implicated in the defense of small-intestinal crypts, only α-defensins (Ouellette and Lualdi 1990; Selsted et al. 1992a; Bevins et al. 1996) have been well documented in humans, mice, and rabbits, while in other epithelial cells β-defensins (Diamond et al. 1991; Bensch et al. 1995; Harder et al. 1997) predominate.

Post-phagocytic degranulation and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation provide the phagocytic vacuoles of PMN with myeloperoxidase and hydrogen peroxide—the catalytic machinery and material needed to iodinate proteins. If radioactive iodine is provided, the contents of phagocytic vacuoles are rapidly radioiodinated. Such phagocytic vacuoles can be recovered from broken cells and analyzed by electrophoresis and autoradiography. In the phagocytic vacuoles of human PMN that ingested *Salmonella typhimurium*, defensins were the predominant iodinated polypeptide (Joiner et al. 1989).

In vitro, defensins display concentration-dependent antibacterial and antifungal activity, and several α-defensins were shown to inactivate herpes simplex virus (Daher et al. 1986). Their potency depends on the primary sequence and cationic charge of the peptide, the microbial target, and its metabolic state, and the composition of the test medium. In particular, the activity of defensins against gram-negative bacteria and many fungi is competitively inhibited by increasing concentrations of physiologic cations such as sodium, potassium, and especially calcium or magnesium, as well as by several serum proteins (Lehrer et al. 1988; Panyutich and Ganz 1991; Panyutich et al. 1994, 1995). Although the available information on the ionic composition of the phagosomal fluid is limited, the concentration of defensins in the phagocytic vacuole is so high that inhibitory ionic factors are likely to be overwhelmed. High defensin concentrations also occur at epithelial surfaces that interface with the external environment, as recently reported for β-defensin 1 in the porcine tongue (Shi et al. 1999).

A similar argument also holds for rabbit alveolar macrophages, phagocytes that contain macrophage cationic peptides (MCP)-1 and -2 (Patterson Delafield et al. 1980). These peptides are structurally (Selsted et al. 1983) identical to NP-1 and -2, the two most cationic defensins of rabbit PMN (Selsted et al. 1984). That MCP-1 and -2 are produced by alveolar macrophages was shown in metabolic labeling experiments with ^{35}S-cysteine (Ganz et al. 1985b) and by their high levels of MCP-1 and -2 mRNA (Ganz et al. 1989). The concentration of MCPs in alveolar macrophages increases during postnatal maturation of rabbit lungs (Ganz et al. 1985b), and is further increased by Freund's adjuvant-induced lung inflammation (Lehrer et al. 1981), so that the amounts of MCPs per cell are similar to the corresponding defensins in rabbit neutrophils. Surprisingly, rabbit peritoneal macrophages lack defensins (Ganz et al. 1989), even when induced by Freund's adjuvant. Thus, the expression of defensins appears to be a marker of alveolar macrophage differentiation in the rabbit. With the exception of rab-

bit alveolar macrophages, no other macrophage in any animal species has yet been shown to contain defensins in amounts comparable to human or rabbit PMN.

Defensin mRNA expression (predominantly bovine β-defensins 4 and 5) has also been detected in bovine alveolar macrophages by polymerase chain reaction (PCR) and Northern blots (Ryan et al. 1998), but cellular concentrations or secretion rates for defensin peptides have not yet been published. Duits et al. recently reported that chimpanzee alveolar macrophages contained mRNA for β-defensin-1 (Duits et al. 2000). In the 1980s, we did not detect defensins in human alveolar macrophage extracts by electrophoretic methods, nor by immunostaining with antibodies against human neutrophil α-defensins HNP1–3. However, human monocytes from peripheral blood do contain detectable HNP1–3 peptides as shown by co-immunostaining with CD14 and anti-HNP1–3 antibody (Agerberth et al. 2000). The concentration and eventual fate of these defensins has not yet been determined.

The recent discovery of cyclic (θ) minidefensins in the leukocytes of a primate, *Macaca mulatta* (the Rhesus monkey), is remarkable for many reasons (Tang et al. 1999; Leonova et al. 2001). These peptides have only 18 residues, including six cysteines that form a three-rung disulfide ladder between its two antiparallel β-sheets. The only known cyclic peptides of animal origin, each minidefensin derives from two peptide precursors (demidefensins), each of which contributes nine total residues to the mature peptide. Although the cellular machinery responsible for splicing and trimming the precursors remains to be defined, it is operational in human leukocytes (Tang et al. 1999). No cyclic minidefensin peptides are known to exist in humans; however, normal human bone marrow expresses an mRNA homologous to the rhesus minidefensin precursors. A synthetic cyclic minidefensin (retrocyclin) whose sequence was based on this mRNA, was remarkably effective in protecting human cells from in vitro infection by HIV-1 (Cole et al. 2002).

4 Cathelicidins

Cathelicidins are a family of mammalian antimicrobial peptides that share a conserved cathelin domain with approximately 100 residues (Zanetti et al. 1995). The active peptide is located C-terminally to this domain, and is in most cases released by proteolysis during the secretion of the peptides from PMN. Like defensins, the C-terminal peptides are broadly antimicrobial but (Turner et al. 1998; Ganz et al. 2000) appear to be less sensitive than defensins to the inhibitory effects of salt and serum. Thus, cathelicidins may have evolved to act in extracellular spaces, where salt and serum components are present in abundance. The function of the cathelin domain is not known with certainty, but it could prevent premature intracellular activation of the potentially cytotoxic peptides, and target the peptide to its subcellular compartment—the specific (or secondary) granules of PMN (Sorensen et al. 1997). The C-terminal peptides

are highly varied, and include α-helical peptides, β-sheet peptides, and other structural forms. The conservation of the cathelin domain encoded by exons 1–3 and the extreme variability of the C-terminal peptide encoded by exon 4 have suggested that this gene family evolved by exon (domain) swapping (Zhao et al. 1995). Cathelicidins are highly abundant and represented by multiple genes in cattle (Romeo et al. 1988; Gennaro et al. 1989; Selsted et al. 1992b) and pigs (Agerberth et al. 1991; Kokryakov et al. 1993; Boman et al. 1993; Storici and Zanetti 1993; Zanetti et al. 1994; Tossi et al. 1995). The number of genes in murine (Gallo et al. 1997), rabbit (Ooi et al. 1990; Zarember et al. 1997; Larrick et al. 1993) and human (Cowland et al. 1995; Larrick et al. 1995; Gudmundsson et al. 1996) is considerably smaller (1–3), but these peptides are relatively abundant. The human cathelicidin LL-37 has been detected in monocytes by immunostaining (Agerberth et al. 2000), but the location and concentration of the peptide in these cells is not known. Cathelicidins have not yet been reported in macrophages, but it is not clear if adequate attempts have been made to detect them.

5
Other Antimicrobial Peptides in Macrophages

The same extractive methods and assays that detected defensins and cathelicidins in PMN of many animal species and in alveolar macrophages of rabbits have also been applied to resting and interferon-γ induced murine macrophages and macrophage-like cell lines. In these studies, antimicrobial peptides similar to those of PMN were not detected, but histones and a ribosomal protein were responsible for the antimicrobial activity of cell extracts (Hiemstra et al. 1993, 1999). Histones in particular have a long history of showing up in screens for antimicrobial substances (Hirsch 1958) and perhaps it is finally time to pay attention to them.

Macrophages have an impressive lysosome system and are keen practitioners of phagosome–lysosome fusion. It is clear that many pathogens that survive within macrophages actively prevent phagosome–lysosome fusion, as shown first for *Mycobacterium tuberculosis* (Armstrong and Hart 1971; Hart 1979). Other organisms now known to subvert phagosome–lysosome fusion in macrophages include *Brucella* spp., *Legionella pneumophila*, *Salmonella typhimurium* and the protozoan, *Toxoplasma gondii*. Interference with phagolysosomal fusion almost certainly prevents some antimicrobial substances from reaching the phagosomes but the nature of these substances is not known.

Although histones are generally thought of as "nuclear" proteins, they are "born" (synthesized) in the cytoplasm, travel to and live in the nucleus, and "die" (undergo proteolysis) within lysosomes (Odaka and Mizuochi 1999). Consequently, the lysosomal compartment of macrophages may contain a brew that is rich in histones and other polycationic nucleoproteins. Exposure to such a mixture could be noxious for many, if not all, ingested bacteria. It still remains to be determined whether histones and other cationic "nuclear" proteins reach

sufficient concentrations in phagolysosomes to contribute to the killing of ingested microbes. If so, it would also be of interest to ascertain whether these proteins enter phagolysosomal compartments during recycling of internal nuclear material, phagocytosis of extrinsic cellular material, or after de novo synthesis induced by cytokines or phagocytosis. As histones and other cationic nucleoproteins have dedicated transport systems for nuclear delivery (Jakel et al. 2002), it would also be of interest to learn if similar pathways exist to deliver them to the phagolysosomal compartment.

6 Summary and Conclusions

Macrophages employ multiple antimicrobial pathways to kill or inhibit phagocytized microbes and microbes that have evolved to parasitize macrophages. Although the pathways that generate reactive oxygen and nitrogen intermediates are similar to those in PMN, there are substantial differences in the oxygen-independent antimicrobial mechanisms. With the possible exception of their juvenile forms (i.e., monocytes) most macrophages lack an extensive primary storage compartment analogous to the granules found in PMNs. Without a storehouse of dedicated antimicrobial peptides and proteins, macrophages appear to rely on alternative mechanisms linked to phagolysosomal fusion to render the environment around ingested bacteria antimicrobial. What effector molecules do macrophages use to kill ingested organisms? If they are polypeptides, then intact and cleaved portions of histones and other highly cationic "nuclear" proteins are the prime suspects. If they are not polypeptides, then an important chapter in macrophage biology (unfortunately, not this one) remains to be written.

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Macrophage Phospholipid Products

Questions on the Synthesis, Secretion and Biologic Effects of Phospholipid Mediators

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Abstract This chapter focuses on a number of elements in the pharmacology, biochemistry, and biology of three families of extracellular phospholipid mediators as they relate to macrophages. In particular, it raises questions that are unique to phospholipids as mediators, including mechanisms of secretion, membrane association, and presentation to their receptors. A number of similarities between the different groups of phospholipids is emphasized, even though the lysophosphatides and PAFs act through seven transmembrane G protein-linked receptors, and phosphatidylserine does not. The phospholipid mediators discussed herein have very broad, and highly potent biologic activities, providing a real motive for appropriate pharmacological manipulation, both in the macrophage and more generally.

Keywords Eicosanoids, Lipid mediators, Lysophosphatides, Macrophages, PAF, Phosphatidylserine, Phospholipids, Receptors

1 Questions on the Synthesis, Secretion and Biologic Effects of Phospholipid Mediators

Macrophages both produce and respond to a host of different types of lipids with an equally bewildering array of physiologic and/or pathologic consequences. The pharmacology of lipid products in relation to macrophage function is thus an immense subject with many potential points of therapeutic attack, from the enzymology of lipid synthesizing and metabolizing enzymes to manipulation of the relevant intracellular and extracellular binding proteins or receptors. Therefore, this chapter will emphasize conceptual issues in lipid pharmacology rather than a catalog of active molecules and their receptors. It will also focus primarily on extracellularly acting phospholipids, in particular the PAF or platelet activating family of phospholipids, the family of lysophospholipid growth factors, sometimes called PLGFs (phospholipid growth-factors), and phosphatidylserines. Each family has broad biologic effects acting via one or more specific cell-surface receptors but with additional complexity related to intracellular metabolism and effects as well as its ability to insert into, and cross, lipid membranes.

The first two groups are generally thought to act in the fluid environment on cell-surface receptors, while PS may mediate its action while remaining on the presenting-cell membrane. However, as we shall see, each of these families may have the potential to act from either location. Surprisingly, although macrophages are known to respond to these bioactive phospholipids, their effects and signaling pathways have not received the degree of investigation that one might have expected.

Phospholipids are the main constituents of cell membranes. Their key structural elements are a glycerol backbone, one or two hydrophobic acyl, alkyl or alky-1-enyl side chains and a phosphate-linked polar head group, usually serine,

ethanolamine, choline, glycerol, or various inositides. They are oriented in the membrane lipid bilayer with the head groups facing the extracellular medium or the cytoplasmic contents and the hydrophobic domains comprising the two leaflets of the lipid bilayer, admixed with other lipidic molecules such as cholesterol and the transmembrane domains of proteins. The phospholipid component of cell membranes is now recognized as being far from an inert matrix for active proteins to float in. Rather it is both a dynamic, highly active environment for such proteins as well as itself providing a critical supply of mediators and signaling molecules for extra- and intra-cellular regulation. These latter functions imply specific binding sites on proteins for phospholipids, a subject of ever-widening interest. Such binding may also be to specific receptors, i.e., leads to signaling activation and cellular responses. In addition, phospholipids can be released/secreted to the outside of the cell and there signal to other cells in the environment via such receptors on the outer cell surface.

Important to our understanding of the complex roles played by membrane phospholipids is the recognition that they are not uniformly distributed in the membrane, either within the plane of the membrane or between its inner and outer leaflet. There is a significant asymmetry between the two leaflets, with phosphatidylcholines (PCs) primarily found in the outer leaflet along with the lipid family of sphingomyelins (SM) or glycosphingolipids. The anionic phosphatidylserine and ethanolamine (PS and PE) are predominantly located in the inner leaflet. As discussed below, this asymmetry will turn out to have important implications in membrane function, lipid secretion and uptake, and cell recognition and stimulation.

Given the size of the subject, the bibliography is representational rather than exhaustive. In many cases, reviews will be cited instead of the original manuscripts, since there may be large numbers of these. Accordingly, I wish to tender my apologies in advance for any seeming omissions. The intent in this chapter is to raise questions, point to potential directions, and suggest future pharmacologic approaches to macrophage lipid mediator production and response.

1.1 The Questions

Detailed consideration of macrophage production and response to bioactive phospholipids raises a number of questions many of which relate to lipid mediators in general and certainly to those belonging to the family of phospholipids. These include: (1) location of the synthesizing enzymes within the cell, (2) mechanisms of transport to the plasma membrane, (3) translocation across the plasma membrane, (4) liberation of the phospholipids from the source cell into the environment, (5) special issues relating to presentation of amphipathic lipids to receptors on the cell membrane of the responding cells, (6) the possibility of direct receptor engagement on the responding cell with surface-membrane phospholipid expressed on the cell of origin and (7) the implications of phos-

pholipid uptake and further metabolism, biosynthesis, and even re-secretion by responding cells such as macrophages.

1.2 The Players

Three examples of externally active, bioactive phospholipid families will form the focus of this discussion: (1) PAF and its analogs, including oxidized PCs of appropriate structure to bind the PAF receptor; (2) Lysophospholipids sometimes called lysophospholipid growth factors and including lyso-phosphatidic acids (LPA), lysosphingophospholipids such as sphingosine-1-phosphate (SIP, sometimes known as SPP) and lysophosphatidylcholines (LPC); (3) Phosphatidylserine (PS). In addition, lipoproteins may also contain members of these potentially active phospholipids. A key point in considering any of these bioactive phospholipids is that all of them can have highly varied structures and in truth represent families of molecules. For the lysophospholipids the *sn1* group can vary. For PAFs and PS, substituents occur in both the *sn1* and *sn2* position. In addition, the structure of PAFs, i.e., *sn1* alkyl or acyl PCs with a short chain *sn2* acyl group (usually acetate) is not strikingly different from lysophospholipids.

As mentioned above, most phospholipids can and do interact with proteins and, as a consequence, exhibit biologic responses, whether these proteins are considered receptors or not. The key distinction here is extracellular action on cell-surface receptors. An additional complexity for phospholipids is their ability to cross the membrane into the cell, there to act on intracellular responding proteins, or to be metabolized into products that have such activities. Elements of this process will only be discussed here in the context of transcellular biosynthesis of the bioactive phospholipids mentioned above. Another huge, and largely unfathomed, possibility is that intracellularly active phospholipids, such as the products of sphingomyelin metabolism (ceramides, sphingosine, sphingosine-1-P, etc.) or the myriad phosphatidylinositol metabolites may gain access to the outside of the cell and act on nearby or adherent cells either on external receptors if present, or more likely, by internalization and interaction with intracellular binding proteins/receptors. Largely ignored so far in this spectrum of potential biologic effects are phosphatidylethanolamines, phosphatidylglycerols, or phospholipids with variations in the *sn1* linkage such as plasmalogens. It seems highly unlikely that, in the long run, these will prove be devoid of extracellular effects and biologic activities.

1.3 Platelet Activating Factor(s) and Eicosanoids

Cleavage of the *sn2* arachidonyl group from PC generates two products, each with enormous potential for further metabolism and biologic function. The hydrolysis is mediated by phospholipases A2, of which the most important for generation of arachidonate products, also known as eicosanoids, is the type IV,

arachidonyl-specific cytoplasmic or cPLA2. The critical role played by this upstream enzyme is exemplified by the phenotypic effects seen in genetically deficient mice (Klivenyi et al. 1998; Fujishima et al. 1999) and in the great interest in its modulation shown at various times by the pharmaceutical industry. The relevance for this chapter is that translocation and activation of cPLA2 is a first step in generation of the myriad eicosanoids, i.e., the subsequent products of arachidonate metabolism as well as being one source for lysophosphatides and synthesis of the phospholipid mediator, platelet activating factor (PAF). These latter are of importance as candidate mediators for a wide range of biologic processes, from fertilization and parturition to inflammation and brain function. PAF has additional historical interest in being one of the first phospholipid mediators to be described and shown to have a distinct receptor.

Eicosanoids are produced following oxidation of the released arachidonic acid by cyclo-oxygenases (COX enzymes or PGH synthases), lipoxygenases, and P450 family enzymes. PAFs are derived from the other product of *sn2* fatty acid hydrolysis, the LPC, by the action of a specific acetyltransferase which inserts an ester-linked acetyl group in place of the original arachidonate. This pathway is sometimes known as the remodeling pathway to distinguish if from de novo synthesis of PAF, the latter being a constitutive process and of less importance in bulk production of the mediator. The initial PC substrate for the $cPLA_2$ can have either an acyl- or alkyl-linked *sn1* fatty acid yielding either acyl or alkyl PAF. The susceptibility of the *sn1* acyl group to lysophospholipase (Nakagawa et al. 1992) or phospholipase A1 activity (including from the cPLA2 itself, which has an additional activity in this regard) makes the alkyl forms more stable and may explain why they were the first to be identified. Increasingly in recent years, it has been recognized that there is an alternative pathway for stimulated PAF production involving the activity of a CoA-independent transacylase (Blank et al. 1995; Winkler et al. 1995). This process may be of particular relevance in the more persistent production of PAF seen in macrophages (Shamsuddin et al. 1997; Svetlov et al. 1997) but, nevertheless does require an initial source of lysophosphatide as recipient of the transacylase process.

Importantly, included in the PAF family are a variety of oxidation products of PCs, since one effect of oxidation of *sn2* unsaturated fatty acids is chain-shortening, i.e., creating molecules with short chain acyl groups that therefore exhibit similar structure and function to PAF (Marathe et al. 1999; Marathe et al. 2000; Marathe et al. 2001) reacting with the same receptor and being susceptible to the same PAF acetylhydrolase. Indeed it has been questioned whether one of the raisons d'être for the plasma acetylhydrolase is to deal with inappropriate concentrations of such oxidized phospholipids, which, for example are found in preparations of oxidized low-density lipoprotein (LDL) (Marathe et al. 2001). The ability of macrophages to mount an oxidative burst is suspected of enhancing this mode of PC modification, i.e., leading to an alternative, non-enzymatic generation of PAF-like activities.

As indicated, macrophages synthesize and "secrete" PAFs (Ninio et al. 1982; Dentan et al. 1996). However, they also synthesize and secrete significant

amounts of PAF acetylhydrolase (Ninio et al. 1982; Elstad et al. 1989; Stafforini et al. 1990) an enzyme that specifically hydrolyses short chain *sn2* fatty acids (including acetate), thereby returning the PAF to lyso PAF. Because of the importance of the alkyl forms of PAF, the term lysoPAF has often been used even though the molecule is directly equivalent to lysoPC containing an *sn1* alkyl linkage. This can then be remodeled, either by re-acetylation back to PAF or by acylation with other, longer chain fatty acids. The presence in macrophage and macrophage supernatants of this enzyme (Ninio et al. 1982; Stafforini et al. 1990) may have led to underestimates in early investigations of PAF production by this cell type. Macrophages are a significant source of plasma acetylhydrolase (Howard and Olson 2000), an enzyme that circulates in association with lipoproteins and has been shown to have significant anti-inflammatory effects in vivo (Stafforini et al. 1987; Prescott 1997; Stafforini et al. 1999). The enzyme is also expressed by macrophages in atherosclerotic plaques (Hakkinen et al. 1999).

1.3.1
Actions of PAFs

PAFs have an extremely broad range of activities and are thought to act primarily through a specific, seven transmembrane, G protein-linked receptor (GPCR), the PAFR (see Henson 2000a,b). To date, only one such extracellular membrane receptor has been identified, although many pharmacological studies have suggested that all the actions of PAF are difficult to reconcile with a single receptor. In addition, as we shall see, possible intracellular effects of this group of molecules needs to be considered, and here too, the likelihood of additional binding molecules or receptors within the cell has been discussed (see Henson 2000a,b; Yamada et al. 1999). Effects on macrophages are complex. Mononuclear phagocytes express the GPCR PAF receptor. Most investigations have suggested the induction of proinflammatory responses including inflammatory mediator synthesis and secretion, as well as effects on motility and weak chemotactic activity for monocytes. One of the intriguing effects of PAFs on inflammatory cells is their ability to induce priming. Here, PAF increases the responsiveness of the cell to other stimuli, even though it does not (or only weakly) initiate the response itself. This priming effect is also seen in macrophages (Kucey et al. 1991; Bautista and Spitzer 1992; Waga et al. 1993; Rose et al. 1995; Bozza et al. 1996; Yamaguchi et al. 1999).

On the other hand, in the context of interaction with apoptotic cells (see below), macrophages appear to respond to PAF with a potential anti-inflammatory effect (Fadok et al. 1998a). Since this was blocked by a PAF receptor antagonist, a potentially complex signaling from this receptor might be suggested. However, one or both of two alternatives may explain these diverse effects. As mentioned, other cell surface receptors for this molecule may exist. As discussed below, PAF may also be translocated across the membrane and there gain access to intracellular "receptors" or binding proteins. One such candidate is the peroxisome

proliferator-activated receptors (PPAR) family of nuclear receptors, particularly PPARγ This has been shown to bind to, and be activated by, oxidized alkyl PC moieties from oxidized LDL (Davies et al. 2001) and could conceivably respond to other members of the "PAF" family. There is separate evidence that PPARγ can mediate anti-inflammatory responses in macrophages (Delerive et al. 2001; Alleva et al. 2002) suggesting a potentially fruitful area for future investigation.

1.4 Lysophosphatides

LPC and LPA contain a glycerol backbone whereas SIP and related family members are lysosphingophospholipids. They all act through closely related, seven transmembrane G protein-coupled receptors including a family of 8 or more EDG receptors for LPA and SIP (Chun et al. 2002) and a newly described G2A receptor for LPC (Kabarowski et al. 2001) or GPR4 receptor for sphingosylphosphorylcholine (Zhu et al. 2001). These molecules are found in significant amounts in the circulation, in association with albumin, other binding proteins, or lipoproteins, and in levels that can vary in different disease states.

Stimulated production of LPA (Moolenaar 1995, 2000) can arise from at least three pathways. In one, phospholipids are hydrolyzed to phosphatidic acid (PA) by phospholipases D (Exton 2000, 2002) with subsequent hydrolysis of the *sn2* acyl group by phospholipases A_2. When the source is membrane vesicles, presumably sPA_2s are responsible (Goetzl and Lynch 2000). In a second pathway, as in activated platelets, diacylglycerol kinase phosphorylates DAG (derived from the action of phospholipase C) to PA with subsequent PLA_2 action to generate LPA. This is released into the medium (see below). The third pathway involves oxidative chain-shortening effects (e.g., on LDL) similar to that seen for non-enzymatic production of PAF-like molecules (Goetzl and Lynch 2000). Similar pathways are operative for the generation of sphingosine-1-phosphate, SIP, starting with sphingomyelinase action on membrane sphingomyelin (Hannun and Bell 1993), but require the final action of sphingosine kinases, very widely distributed, stimulatable, intracellular enzymes located in both the cytosol and membrane fractions of cells (Liu et al. 2000). To no great surprise, the intracellular levels of SIP are highly regulated, in part by the actions of phosphohydrolases (Le et al. 2002).

LPC can be produced by the action of any of the many phospholipases A_2 that can act on PC. In the cell, this can include the calcium dependent $cPLA_2$ that is so important for eicosanoid generation as well as calcium-independent enzymes. However, as already discussed, the presence of reacylating enzymes would be expected in general to result in rapid removal of the LPC. In fact, the relatively high levels of acetyl-CoA in the cell and the presence of an acetyltransferase (see above) has even led to the suggestion that intracellular PAF production might be, in part, a mechanism for rapid removal of potentially toxic LPC. (LPC acts as a potent detergent at higher concentrations.) Extracellular $sPLA_2$s from multiple sources (including from infectious organisms) acting on PC in

membrane vesicles, damaged cells, lipoproteins, etc. can all initiate LPC production. Macrophage-derived $sPLA_2$ would be a likely source in many circumstances including, for example, in LPS-induced LPC generation in the lung (Arbibe et al. 1998).

As expected from the discussions above, LPC has also been suggested to arise from oxidation of PC-containing *sn2* unsaturated acyl groups, for example of LDL. A cautionary note here is the distinction between chain shortening, leading to generation of short-chain *sn2* acyl groups, which would act like PAF on the PAF receptor, versus the generation of true LPC. In even more general terms, a clear comparison between the PAF and lysophosphatide family of lipids in terms of production and receptor-driven effects has still to be completed. To this point, the two literatures have tended to remain separate.

Initially considered as having possible ionophore-like activity, LPA soon became recognized as a ligand for receptor-mediated calcium mobilization (Moolenaar 2000) and a host of other cellular responses including cell replication, cytoskeletal regulation, and movement or inflammatory mediator generation. This broad spectrum of activities extends to SIP and LPC (see Moolenaar 2000; Tigyi et al. 2000; Hla et al. 2001; Graler and Goetzl 2002; Spiegel and Milstien 2002). These molecules also exhibit a wide spectrum of intracellular roles (see for example: Hla et al. 1999). Actions on the immune system and inflammation have not received as much attention as on tissue cells but are likely to be important (see Graler and Goetzl 2002). LPC has been suggested to have varying effects on lymphocytes, and genetic deletion of the LPC receptor G2A resulted in progressive inflammation and a lupus-like syndrome (Le et al. 2001).

Roles for these mediators in macrophage function are significantly understudied. However, macrophages do express a number of the EDG receptors (Goetzl et al. 2000a; Hornuss et al. 2001; Lee et al. 2002). LPC has been reported to have chemotactic activity on monocytes (Quinn et al. 1988) and the broad abilities of the lysophospholipid mediators to affect cell movement (Spiegel et al. 2002b) (in part through actions on the *rho* family of GTPases) suggest at least one likely group of effects.

LPA and SIP likely act as proinflammatory stimuli and appear able to enhance inflammatory mediator production (Lee et al. 2002). On the other hand, LPA activation of adenylate cyclase has been reported in RAW cells (Lin et al. 1999) raising the possibility of more complex effects. LPA induces calcium mobilization in microglial cells (Moller et al. 2001). It has also been suggested to function as a macrophage survival stimulus acting through phosphoinositol (PI)3 kinase and presumably Akt (Koh et al. 1998). These authors raise the possibility that LPA may explain some of the known survival effects of serum (plasma). LPC can also activate PKCs in macrophages (Prokazova et al. 1998), enhances FcR-mediated phagocytosis (Morito et al. 2000), and when injected into the spinal cord, initiates macrophage accumulation (Ousman and David 2000), although whether by direct or indirect effects is not clear. In general, the full spectrum of activities, likely autocrine and paracrine effects, regulatory processes, and in vivo biologic relevance for the macrophage remain to be determined.

1.5 Phosphatidylserine

PS is normally found in intracellular membranes and on the inner leaflet of the plasma membrane. As discussed below, when it becomes expressed on the outer leaflet, especially in apoptotic cells, it serves as a recognition signal for removal of such cells. A specific receptor (PSR) has been described that recognizes the polar head group of PS in a stereospecific fashion and which mediates this activity (Fadok et al. 1998b, 2000, 2001a,b). On the other hand, PS is recognized by a host of proteins, both within and without the cell. For example, many of the intracellular signaling proteins, such as most of the PKCs use PS as a co-factor. Many of the annexin family of molecules also bind PS, and annexin V has become a standard marker for detecting the phospholipid on the cell membrane. In the extracellular environment, coagulation factors V and X bind and use PS to accelerate clotting. Molecules such as GAS-6, β glycoprotein 1, MFG-E8, members of the collectin family (see below) and probably many others are known to interact with PS. Scavenger receptors can also bind anionic phospholipids, including PS. In many cases, unlike the PS receptor mentioned above, these binding proteins do not show high specificity for PS and will also bind PE, PI, or PA. On the other hand, this does not diminish their potential role in recognizing and responding to PS in the environment or on cell surfaces.

A significant source of PS in the extracellular environment are membrane vesicles. Activation of platelets results in external PS expression and concomitant assembly and activation of the coagulation cascade. It is now recognized that liberated vesicles are the major source of this activity. Most cell types, including mononuclear phagocytes, actively release vesicles and do so to a greater degree when activated. As discussed below, we suspect a high degree of PS expression on such structures. Membrane fragmentation during cell death (necrosis or post-apoptotic cytolysis) also results in vesicle formation and, without the normal mechanisms for regulating phospholipid asymmetry (see below) they are likely to exhibit PS on their surfaces.

Macrophages can expose PS on their surface during stimulation, phagocytosis, and apoptosis. They express the PS receptor (Fadok et al. 1992, 1993, 1998b, 2000) and respond to PS binding of this by ingestion (for example of the apoptotic cell or membrane vesicle) through, what we suggest is a process of stimulated macropinocytosis (Hoffmann et al. 2001). Ligation of this receptor also induces the generation of anti-inflammatory molecules and signals and blocks the production of proinflammatory chemokines, cytokines, growth factors, and eicosanoids (Fadok et al. 1998a).

In summary, then, macrophages make and respond to PAFs, LPCs, and LPAs. They can express PS on their surfaces, probably release PS-expressing vesicles as well as recognize and respond to PS on other cells and vesicles. They also can both make and respond to a wide variety of eicosanoid types, including prostanoids, HPETES and HETES, leukotrienes (LTB4 and sulfidopeptide leukotrienes), lipoxins, and isoprostanes. This clearly shows the breadth of expression

of both the appropriate synthesizing enzymes as well as of surface receptors for the products; although, not surprisingly, different macrophage populations may express different patterns of these and may upregulate or downregulate them to different degrees to different stimuli.

As noted, there are a number of additional common features exhibited by these families of bioactive phospholipids. They each have significant intracellular signaling and co-factor roles as well as exhibiting specific extracellular receptors. They can be found in lipoproteins and may therefore play important additional roles in atherogenesis. Intriguingly, antibodies against these phospholipids (LPCs, PS, etc.) are not uncommon, particularly in autoimmune disease.

2 Sites of Synthesis

Phospholipid synthesis is segregated to different sites within the cell depending on the lipid species being generated (Voelker 2000). There is also a large body of work addressing membrane biosynthesis, which does not need to be considered particularly here. Most of the plasma membrane PS in the resting cell ends up on the inner leaflet. More to the point for our discussions is the site at which the biologically active forms of the externally acting phospholipids are generated.

It is now recognized that many of the key synthetic enzymes for these mediators translocate to, or are located at, the nuclear membrane, endoplasmic reticulum, or Golgi. This includes the initiating $cPLA_2$, as well as the PGH synthases involved in prostanoid generation and the 5-lipoxygenase (5-LO) required for synthesis of leukotrienes. The acetyltransferase involved in PAF synthesis is also thought to be found at these intracellular sites (Record and Snyder 1990; Samples et al. 1999). The co-localization of these various enzymes would certainly make for enhanced efficiency of product generation, particularly if the lipid substrates and intermediates are inserted into, or bound to, hydrophobic membrane domains at this one site. On the other hand, location on what is presumed to be the cytoplasmic face of nuclear or endoplasmic reticulum membrane does raise questions about transport of products and intermediates from this site to the plasma membrane, not to mention passage across this to the outside environment (see below).

Secretary phospholipases A_2 can, under specialized conditions, act on phospholipids of the external membrane to generate lysophosphatides and macrophages synthesize and secrete $sPLA_2$s including those of groups II, V and X. (Berger et al. 1999; Morioka et al. 2000; Jaross et al. 2002) Some of these show preference for PS or PE, but others may have broader specificity. In a number of cell types, including macrophages, there is also evidence for complex functional coupling between $sPLA_2$ and $cPLA_2$ (Balsinde et al. 1998) although this may not apply to all types of macrophage or stimuli (Dieter et al. 2002). Whether these externally acting enzymes can generate bioactive LPA or LPC [or lyso-PAF for

later internalization and conversion to PAF at the endoplasm reticulum (ER)] is not clear but must certainly remain a possibility. Intracellular PLA_2s must be seen as likely candidates for the generation of the bioactive lysophosphatides and these may include those with varying *sn2* fatty acid specificities, implying varying degrees of accompanying arachidonate release and eicosanoid synthesis. One poorly studied or considered question is a possible source of bioactive phospholipids from intracellular organelles (granules, vesicles, etc.). Once again, the issue of local generation of active phospholipids at intracellular membranes must raise questions of access to the plasma membrane and to the outside environment.

3
How Do Intracellularly Generated Bioactive Phospholipids Gain Access to the Plasma Membrane and Extracellular Environment?

The bulk of cell phospholipid is found in membrane bilayers, in intracellular membranes and organelles or in the plasma membrane. However, in order to supply biologically active phospholipids to the external environment, most of these molecules must transit one or more membrane bilayers. Initially synthesized in the ER, often modified enzymatically subsequently, the lipid elements of membrane assembly are a subject of detailed ongoing investigation. Transit across the cytoplasm to the cell membrane is clearly required not only for maintenance of the membrane itself but also for "secretion" of the bioactive phospholipid mediators. Access of these to the external environment may come from three main processes. In one, conventional secretion involving vesicle (or granule) transport to the plasma membrane with discharge of contents would cause release of any contained phospholipids. It would also lead to surface expression of phospholipids inserted into the intraluminal membrane leaflet of the vesicle or granule.

In a second process, phospholipids may be transported from synthesis or modification sites within the cell to the inner surface of the plasma membrane, probably in physical association with transport proteins. Insertion into this inner leaflet would leave the phospholipid available for regulated transmembrane movement (translocation) to the outer leaflet, a process sometimes called "flop" to contrast with inward movement across the membrane ("flip") or bidirectional movement (flip-flop or "scrambling"). Fusion of transport vesicles with the inner surface of plasma membranes would also supply the inner leaflet of the latter with new phospholipids that could later be translocated to the outer surface.

A third potential mechanism for supply of extracellular phospholipids is more complex and could include elements of each of the former. Many cell types in vitro release membrane vesicles from their surface, both spontaneously and in response to stimuli. There is also increasing evidence for the presence of such vesicles in the circulation, and such vesicles may contain oxidized phospholipids with biologic effects on monocytes (Huber et al. 2002). Stimulated monocytes, for example, release large numbers of such vesicles and these have been shown

to contain protein mediators of inflammation (MacKenzie et al. 2001). Such vesicles may also supply externally expressed bioactive phospholipids to receptors on other cells. The mechanisms for vesicle release are only now beginning to be studied. Teleologically, over-supply of plasma membrane from secretory events would need to be regulated to maintain cell size and surface area. This could be achieved both by re-internalization of the extra membrane or its pinching off and release of vesicles to the environment. It seems likely that the vesicles could also act as stimuli to adjacent or even distant cells.

As far as PAF and lysophosphatide mediators is concerned, the exact mechanisms of such transport are not known. Intracellular PAF binding proteins have been described in macrophages and could serve as transporters (Banks et al. 1988; Lumb et al. 1990). The phosphoinositol transfer proteins (PITPs) also carry single molecules of PC (as well as sphingomyelin and phosphatidic acid) and exchange them for membrane-resident phospholipid molecules (Li et al. 2002). They might serve similar roles for the bioactive phospholipids under discussion. PAF or lyso-PAF appear able to move efficiently across the cytoplasm in both directions, since radiolabeled compounds can be seen in the nuclear membrane by autoradiography very soon after addition to the outside of the cell (F.H. Chilton, P.M. Henson and R.C. Murphy, unpublished observations). This could certainly fit a transport protein model. On the other hand, the molecules are relatively hydrophilic, so simple diffusion cannot be ruled out. The possible presence of PAF, or lysophosphatides, in intracellular transport vesicles has not received much attention, but there seems no intrinsic reasons why this could not also serve as a route for export.

4
How Do Bioactive Phospholipids Cross the Plasma Membrane?

This question of how bioactive phospholipids cross the plasma membrane is important for both secretion and surface expression of active phospholipids as well as for uptake and then re-expression in transcellular biosynthetic processes. Studies with PAF have shown that this molecule can be actively transported across the plasma membrane in both directions. At issue for such a process is insertion into one leaflet of the bilayer followed by a mechanism for flip or flop (inward or outward) to the other leaflet. Compounding factors are the possibility that the phospholipid might be metabolized at the membrane and/or may also be interacting with proteinaceous membrane receptors. PAF proved useful as a tool for understanding membrane phospholipid movement, in part because the *sn1* ether linkage is resistant to phospholipase cleavage. Short-chain *sn2* analogs that were biologically active but also resistant to hydrolysis were additionally helpful (Bratton et al. 1991, 1992; Bratton 1994).

Phosphatidylcholines with long-chain fatty acids are only poorly moved across the membrane bilayer, but those with shorter *sn2*-linked groups moved more rapidly (Zhou et al. 1997). However, in both cases, translocation is enhanced after activation of the cells. In the case of PAF, this activation can arise

from ligation of the PAF receptor itself, so that stimulation through this receptor can enhance non-selective uptake of PAF or other phospholipids that have become inserted into one or other membrane leaflet. While uptake of PAF may occur through internalization of the ligated receptor, this appears to represent only a small proportion of total uptake; most presumably occurs through the action of flippases. Thus, blockade of the specific PAF receptor but ligation of unrelated G protein-linked receptors can induce just as much uptake.

4.1 Phospholipid Scrambling

The physical processes involved in phospholipid translocation across the plasma membrane are unknown. However, a group of phospholipid "scramblases" appear to be required (Williamson et al. 1995, 2001; Comfurius et al. 1996; Zhou et al. 1997; Frasch et al. 2000; Sims and Wiedmer 2001). The name derives from their ability to move phospholipids bidirectionally across the membrane, thereby reducing the inherent phospholipid asymmetry between the inner and outer leaflets, i.e., scrambling the membrane. The activity seems to be significantly independent of phospholipid head group and, as suggested above, probably acts more efficiently on molecules with shorter *sn2* substituents. It has been suggested that scramblase activation requires PKC-dependent phosphorylation and the presence of calcium for optimal activity (Frasch et al. 2000). There may also be a tyrosine phosphorylation event involved (Sun et al. 2001). Scramblases are a family of type 2 transmembrane proteins whose exact contribution to the phospholipid movement remains to be determined. It is hard to see how they can directly mediate this process. Assembly into multimers in the membrane might lead to local disorder in the lipid bilayer to enhance movement. More likely, other membrane proteins are involved. Possible candidates for such are members of the ATP cassette family of proteins already known to participate in phospholipid and cholesterol import or export.

Regulation of membrane phospholipid distribution is suggested to be important and requires some concerted investigation. The lipid environment is increasingly seen to play a critical role in the function of membrane proteins, receptors, etc., and the activity of phospholipid translocation processes is bound to contribute to this and the overall cell functions. A recently emphasized case in point is the recognition of proteases that appear to function within the hydrophobic domain of the lipid bilayer (Wolfe and Selkoe 2002). Activation of many, or even most, cell types (i.e., activation of PKCs and mobilization of calcium) leads to transient membrane phospholipid scrambling. The relationship of this to defined membrane domains (e.g., cholesterol-rich regions, rafts, caveolae, etc.) remains to be clarified, although early evidence suggests ties between rafts and scrambling (Kunzelmann et al. 2002).

Since resting cells, including macrophages, maintain membrane phospholipid asymmetry, casual activation of scramblase and scrambling must generally be transient, implying both a cessation of the scramblase activation and a recti-

fication process to return any externalized PS or PE back to the inner leaflet. Although different cell stimuli induce scrambling at different rates and for different lengths of time [G protein receptors, fast and shorter, or growth factors such as macrophage colony-stimulating factor (M-CSF), slow and longer], the process is usually time limited. Although not formally shown at this point, cessation of scrambling may be attributed to the action of phosphatases on the scramblase, inactivation of the PKCs, and/or rectification of the increased calcium levels. However, cessation of the actual scrambling is not enough to maintain homeostasis, the translocated phospholipids are actively returned to their original state. This is achieved by an aminophospholipid translocase. Its activity requires ATP and is blocked by high calcium. Various candidates for this activity have been implicated. A possible aminophospholipid translocase (a member of the P-type ATPase family) is widely distributed from plants to man (Chen et al. 1999; Daleke and Lyles 2000; Ding et al. 2000; Gomes et al. 2000) and although the validity of this candidate has been challenged, more recent data in *Arabidopsis* does add support to an aminophospholipid translocase role for these P-type ATPases (Gomes et al. 2000).

While alterations in phospholipid scrambling have been shown to be important in maintaining or altering phospholipid asymmetry, in surface expression of PS, and in "secretion" of PAF, there has been little attention to a role for release of the lysophosphatide mediators. The comment is made in Moolenaar (2000) that "Precisely how LPA and SIP are released into the extracellular environment remains to be elucidated." We are suggesting that translocation across the membrane mediated by activated phospholipid scramblase as outlined for PAF is an important first step. This would be followed by partitioning onto carrier proteins such as albumin or lipoproteins (see below); this may represent a key release mechanism for these phospholipids as it appears to be also for PAFs.

4.2
Membrane Scrambling and Phosphatidylserine Expression in Apoptosis

By contrast, permanent membrane scrambling is seen in cells that are undergoing the process of apoptosis. In this case PKCδ is cleaved and rendered permanently active by the action of caspase 3, increased calcium levels are maintained, and the aminophospholipid translocase activity is inhibited probably as a consequence of the decreased ATP and increased calcium. This process leads to permanent expression of phosphatidylserine on the apoptotic cell surface now known to be important for apoptotic cell recognition and removal as well as for the biologic consequences of this (see below). As mentioned above, while much emphasis has been placed on PS in this circumstance, scrambling of the membrane phospholipids in apoptosis is likely to have other profound effects on the cell surface. This would range from alterations in other phospholipids (internalization of sphingomyelin, externalization of PE), alterations in lipid and protein orientation and distribution in the membrane, effects on rafts, etc. It also proba-

bly contributes to other effects seen in apoptosis, such as membrane blebbing, cell shrinking, binding of recognition molecules such as collectins, thrombospondin, β-glycoprotein 1, GAS-6 (Scott et al. 2001), MFG-E8, (Hanayama et al. 2002). Expression of PS on apoptotic membrane blebs enhances local pro-coagulant activity (Casciola et al. 1996). The increased membrane scrambling that occurs during apoptosis would also be expected to move any internal phospholipid mediators to the outer membrane, which could include PAF and the lysophosphatides. Apoptosis is often accompanied by PLA_2 activation, which could increase the supply of such mediators.

Macrophages are not at all unique in their apoptotic mechanisms or manifestations. Although not studied as much as many other cell types, they too express PS on the membrane and are certainly removed as efficiently as other cells when they become apoptotic in vivo.

4.3
Why May Membrane Phospholipid Translocation Be Important?

The maintenance of phospholipid asymmetry is suggested to play a major role in membrane functions, with regard to the structural and biochemical effects of the lipids themselves as well as for attached or embedded proteins. The role of surface PS expression during macrophage activation and phagocytosis is not yet delineated but is expected to contribute to the processes in some fashion. Intriguingly, such PS exposure has been reported on the macrophage during uptake of apoptotic cells, i.e., during a process that involves PSR recognition of PS also on the target (Marguet et al. 1999; Callahan et al. 2000).

As argued herein, we also suggest an important role for phospholipid scrambling in supplying new phospholipids to the extracellular environment, for access to other cells, or "release" into the surroundings. However, the bidirectionality of the process also suggests a role in uptake of these same phospholipids, especially the lysophosphatides and PAFs with the opportunities for intracellular metabolism, inactivation, or conversion, or even intracellular biologic effects.

Whether there are any pharmaceutical possibilities in altering scrambling and/or aminophospholipid translocase activity during apoptosis is as yet unclear. However, as we begin to understand the processes better, as well as their consequences, such potential may become apparent.

5
How Are Phospholipids Released from the Membrane into the Aqueus Extracellular Milieu?

Bioactive phospholipids that have reached the external membrane leaflet of the originating cell may act on other cells in the environment by release into the medium, blood, or interstitial fluid or because they are directly recognized by receptors on the responding cell. Here we will consider the release aspects.

5.1 Carrier Proteins

Even relatively hydrophilic phospholipids such as LPC may not easily partition from the membrane into an aqueus environment without some help, and the longer the *sn2* fatty acid, the more difficult this process will be. Extracellular binding proteins, particularly albumin, are more probably involved in all these process. Serum albumins have a high capacity for lipid binding on multiple sites and with varying affinities. This makes them ideal as carriers for biologically active lipids. A specific example is the confusion seen in the early studies of a platelet-activating material released from IgE-stimulated blood or leukocytes when the experiments were carried out (as they originally were) in protein-free medium. The inclusion of albumin in the medium immediately enhanced the activity, its stability, and biologic effects, not to mention setting the stage for its isolation and characterization.

However, while simple to state, this issue adds enormous complexity to an understanding of the effects and activities of phospholipid mediators, especially in vivo. Seldom are in vitro experiments carried out in 100% plasma. Addition of 2% or even 10% plasma, serum, or albumin hardly mimics real-life conditions. Albumin in vivo will already have bound lipids so that the surface phospholipids of interest will have to displace these before being "extracted" from the membrane. Partition onto albumin will depend then on many factors: the protein concentration, relative affinity of the phospholipid for given binding sites, whatever phospholipids are already on these sites as well as the hydrophilicity of the phospholipid in question (after all, the albumin itself is a globular, water-soluble molecule). An ability of albumin to physically bind to the cell membrane would contribute to this process. While many studies have shown how difficult it is to completely free isolated cells of albumin, this may be in part due to internalization in microvesicles as well as surface attachment. Might such attachment itself be due to phospholipid binding (see below)?

In vitro stimulation of macrophages, harvesting the supernatant and subsequent examination of active phospholipid effects on responding cells will be critically dependent on all these issues.

In order to further illustrate this point, standard assays for phospholipid translocation across the plasma membrane involve incubation of the cells with lipid (usually supplied on albumin, in micelles or liposomes) to allow insertion into the outer leaflet and translocation. Then, in order to detect intracellular phospholipid, any material remaining on/in the outer leaflet is back-extracted with lipid-free albumin. Not usually considered in such studies is how much of the preexisting extracellular leaflet phospholipid is also extracted by such a procedure. This is probably not of critical importance when investigating translocation in this way. However, the issue does raise intriguing questions about ongoing phospholipid exchange from membrane to albumin and back in tissue culture or even in vivo. When macrophages or other cells express PS on their surface during activation (transient) or apoptosis (permanent), is any of this por-

tioned onto albumin to become potentially available for PS receptors on other cells?

This discussion has focused on albumin in part because of its high capacity and low specificity for lipid binding as well as its high concentration. LPA and LPC in plasma are found bound to albumin. However, there may well be other, more specific carriers in plasma or tissues that play more selective roles. One might wonder, for example, whether the acute phase reactant, C-reactive protein (CRP), which is known to bind phosphorylcholine, could "extract" and carry LPC from a producing cell to one with LPC receptors. Gelsolin has also been suggested as a carrier for lysophosphatides (Goetzl et al. 2000b), particularly perhaps after cellular damage or inflammation. Clearly, the other major source of carriers for bioactive phospholipids are the lipoproteins. The issues of partitioning from membrane to carrier apply here as well and once again from an experimental perspective, few investigators carry out mediator experiments in the presence of physiological levels of lipoproteins. Plasma phospholipid transfer protein (PLTP) exchanges phospholipids between lipoproteins (van Haperen et al. 2000; van Tol 2002), but whether it is important in movement and distribution of the bioactive phospholipids that we are discussing is not yet clear. The presence of PAF acetylhydrolase in lipoproteins raises the intriguing likelihood that it serves here to limit the presence of PAF or bioactive, oxidized, PCs in this circulating pool. An implication might be to confine the effects of highly active PAFs to local sites.

5.2 Vesicles

As discussed above, the other way in which bioactive phospholipids could be "released" from cells is as components of vesicles. The presence of bioactive phospholipids in the membranes of such vesicles would also be expected to allow stimulation of their cognate receptors (see below). Whether vesicles released from monocytes or macrophages do indeed contain such phospholipids remains to be determined. Vesicles and apoptotic bodies liberated during apoptosis also express PS. It seems highly likely that vesicles liberated from activated macrophages do the same and that these could possibly, in the right circumstances, provide a potential stimulus to cells via the PS receptor. Vesicles containing PAF and/or lysophosphatides might be able to directly stimulate the PAF or EDG receptors on target cells.

5.3 Retention or "Storage" of Bioactive Phospholipids

An early observation in the study of PAF synthesis was that in most cell types, relatively little PAF was actually released into the medium. This cellular "retention" has led to questions about intracellular activities of the molecules. We suspect that it reflects in part the combined need for the newly synthesized mole-

cules to be transported across the cytoplasm, translocated across the plasma membrane, and partitioned onto carriers in the environment. In like fashion, the lysophosphatide mediators are also sometimes seen retained or "stored" within cells (e.g., platelets) (Yatomi et al. 1997; Goetzl and Lynch 2000). Whether these molecules are ever stored in vesicles or granules for later export is not yet clear.

6
How Do Phospholipids Stimulate Surface Receptors?

6.1
Presentation

A number of special issues arise in consideration of the way in which a phospholipid might interact with its surface protein receptor.

6.1.1
Albumin and Carrier Proteins

Solubility in the aqueous environment is just as important here as in release from the cell of origin. It is probably no accident that the phospholipid mediators under discussion (lysophosphatides, PAF, etc.) have significant hydrophilic properties in comparison with the bulk of their membrane counterparts. However, it seems likely that even here, presentation on carrier proteins, especially albumin, is the norm. How much is ever free in the aqueous milieu is questionable. This point has certainly been emphasized (and challenged) for interaction of PAF with its receptor (Clay et al. 1990; Grigoriadis et al. 1992). We suggest that in biologic fluids, PAF is bound to one of four potential binding sites on albumin and exchanges from these to the receptor which has a higher affinity for the molecule. Once again, at least one implication from this would be potential competition for PAF on the albumin by other lipids, since the binding sites are not specific. In the circulation, the concentration of albumin (lipid-binding capacity) is high, but in the tissues and in the immediate environment of the macrophage membrane this may not always be the case. In vitro stimulation experiments are seldom carried out in whole plasma. The degree to which LPA or LPC are presented to their receptors on albumin or other potential carriers is unknown, but it seems highly likely from their related structures that the same issues apply.

6.1.2
Membrane Insertion

If not on albumin, the PAF is susceptible to metabolism by plasma PAF acetylhydrolase (probably following partition into lipoprotein particles) and incubation with plasma leads to rapid inactivation. In the presence of cells, the bioac-

tive phospholipids also rapidly and effectively insert into the outer leaflet of plasma membranes. As we have already noted, if the cell is actively scrambling its membrane, the material will then be flipped to the inside with consequences that can include metabolism, remodeling, and even possible direct effects on intracellular signaling pathways. Transcellular metabolism of PAF, lyso-PAF or analogs appears to result from uptake subsequent to membrane insertion rather than following receptor binding. However, this question has not been easy to resolve. PAF receptor antagonists can certainly prevent internalization through the receptor and do not block initial insertion into the outer leaflet of the cell membrane. On the other hand, stimulation of the cell through ligation of the receptor enhances internalization (see above) and often activates or alters the synthetic or metabolic processes within the cell so that antagonist blockade of transcellular metabolism can occur at this step. Examination of transcellular metabolism (see Sect. 7) in the presence of specific PAF receptor antagonists but also with alternative stimuli (i.e., active on unrelated receptors) has helped answer these questions.

Insertion into the membrane too provides a competitive site for the phospholipid mediator interaction, although the affinity will be much lower than that for the receptor. The additional question is whether membrane inserted PAF or lysophosphatides can act on their surface receptors from this site or must be "released" or rebound to albumin to have this effect. Can the membrane act as a store for immediately adjacent (and relatively protected) ligand that contributes to the overall effects of such phospholipid ligands on the receptor?

It is clear that these considerations might play havoc with conventional analysis of receptor–ligand interactions. It is unlikely that these types of molecules are ever in true monomeric form. If not in membranes or on proteins, they tend to interact with themselves or other lipids to form micelles and indeed, in all too many in vitro experiments, appropriate attention to the critical micellar concentration (i.e., the physical form of the ligand) appears to be lacking. Another confounding feature is that insertion of these hydrophilic (amphipathic) phospholipids itself induces physical changes in the membrane, in high enough concentrations, even causing cell lysis. This demands experimental studies with low concentrations of ligand. However, when a cell is itself releasing lysophosphatides to a nearby recipient in vivo, or even in vitro, the local concentrations are not usually known and, especially if released in vesicles, could reach levels with possible physical effects on portions of the responding cell membrane.

6.2
Membrane Presentation

At issue here is whether phospholipid "mediators" in the outer leaflet of cells can, from this site, interact with cognate receptors on another cell. A special case would be presentation from membrane vesicles to receptors on the responding cell.

6.2.1 Phosphatidylserine

Specific recognition of, and response to, PS on the surface of apoptotic cells represents a clear case of this type of membrane presentation. A receptor for PS has been identified (Fadok et al. 2000) that recognizes the polar head group of the phospholipid in a stereospecific fashion. Numerous other extracellular proteins and surface "receptors" are also known to bind PS (see above). In particular in macrophages, these include a variety of scavenger receptors. The extracellular, soluble, PS-binding molecules may serve to cross-link apoptotic cells or membrane vesicles to macrophage surfaces and, thereby, induce or enhance uptake and removal.

We have suggested that the PS receptor plays particularly important roles in such removal. Its blockade or removal prevents much of the apoptotic cell uptake into macrophages in vitro and preliminary evidence supports such a role in vivo as well (Hoffmann et al. 2001; Huynh et al. 2002). More indication of the in vivo importance of this receptor will come from studies of its knockout, which is not yet available. On the other hand, blockade of the PS by attachment of annexin V both in vitro and in vivo has been shown to prevent uptake of apoptotic cells and some of the consequences of this (Bennett et al. 1995; Blankenberg et al. 1998; Stach et al. 2000). Interaction of apoptotic cells with, and uptake into, macrophages does not induce the proinflammatory consequences usually associated with phagocytosis. Rather it results in an active suppression of proinflammatory mediator production (Fadok et al. 1998a). This appears in part due to selective induction of anti-inflammatory mediators such transforming growth factor (TGF)-β. Under some circumstances there is also increased interleukin (IL)-10 production (Voll et al. 1997; Fadok et al. 2001c). These effects appear to be due to the PS receptor since in its absence, or blockade, the anti-inflammatory response is no longer seen. Direct ligation of the PSR with antibody or PS liposomes induces these molecules, is anti-inflammatory and, in vivo, can hasten resolution of an inflammatory response.

Intriguingly, engagement of the PS receptor suppresses proinflammatory eicosanoids (e.g., thromboxanes) and enhances production of PGE_2 and PGI_2 (Fadok et al. 1998a; and W. Vandivier, unpublished observations). In addition, some of the in vitro suppression of proinflammatory mediator production was blocked by COX inhibitors (Fadok et al. 1998a). Selective effects of the PSR on eicosanoid biosynthetic enzymes are implicated. Increasingly, evidence is appearing of subversion of these apoptotic cell recognition systems and their anti-inflammatory consequences by parasites. Thus *Leishmania* may use the PS receptor to interact with macrophages and alter inflammatory reactions (de Freitas Balanco et al. 2001) and *Trypanosoma cruzi*, the PGE_2 production to evade the inflammatory response (Freire-de-Lima et al. 2000). In addition, and so far unexplained, pharmacologic blockade of the PAF receptor also reduced the anti-inflammatory effects of apoptotic cell uptake by macrophages, and this could be mimicked by direct addition of PAF to the cells. While production of

PAF from macrophages interacting with apoptotic cells or PSR ligands could not be detected, this may have been because of low levels of free mediator and/or its attachment to any of the membranes in the system. A potential dual pro- or anti-inflammatory role for PAF and its receptor on macrophages is raised by these observations but requires much more investigation to prove.

At issue here is how PS in the membrane leaflet is bound by the receptor on a responding cell. At least the receptor recognizes the polar head group of the PS and this is facing the aqueous, extracellular environment. (The potential role of other substituents of the PS molecule in PSR binding are at this point unknown.) On the other hand, it would not seem easy for a protein in a cell membrane surrounded by surface carbohydrates, etc. (the glycocalyx) to gain access to the head group of a phospholipid on the cell being recognized also surrounded by surface structures. In general, cell membranes are mutually repulsive unless specific adhesion molecules are engaged. We have suggested a two-part process in recognition and removal of apoptotic cells in which adhesion or tethering ligands play a key role in bringing the two players in close apposition and, we propose, in allowing appropriate engagement of the PS receptor (Hoffmann et al. 2001). Either intrinsically, or because of these geographical constraints, it would appear that the PSR is of low effective affinity, although whether this is true in any real Michaelis Menton sense is completely unknown. Local high densities of PS on membrane blebs or on free vesicles would also serve to enhance the potential for PS receptor activation.

The potential difficulty for PS receptor activation in "normal" circumstances may have biologic implications in that it would mean that transient PS exposure on activated cells would not initiate responses in adjacent macrophages unless a number of other factors also came into play, including the presence of key tethering ligands and local high concentrations of "aggregated" PS on the target cell. Soluble PS-binding proteins acting as bridge molecules might have less of a problem gaining access to the surface phospholipids but again may need other factors or local high surface densities to be effective back on the responding cell.

6.2.2
Surface Effects for PAF and Lysophosphatides

How much any of these potential constraints apply to the other bioactive phospholipids is not at all clear. Their short (or absent) *sn2* fatty acids and higher hydrophilicity means that liberation from the membrane is easier (see above). Numerous studies with PAF analogs and antagonists suggest that the active site in the receptor can "see" each portion of the molecule, i.e., the phosphorylcholine, short chain *sn2* substituent, and the *sn1* group, an alkyl link in this position being more effective. The clear implication is that ultimately the PAF that acts on the receptor is completely free of the membrane of origin or carrier molecule. Can the receptor "extract" the PAF from a cell membrane? Is membrane-associated PAF bioactive? In this regard, an important series of experiments has

addressed the effects of endothelial PAF on monocyte adhesion and stimulation (Patel et al. 1993; Zimmerman et al. 1996). Here PAF on the endothelial membrane can stimulate the monocytes. Adhesion molecules are also involved, perhaps to enhance binding to the PAF receptors. What is not clear is whether the PAF is actually membrane-inserted in this circumstance or is bound to an endothelial surface protein serving to "present" the molecule to the responding monocytes.

Whether similar phenomena and constraints occur in the case of SIP, LPA, LPC, or even PS is not yet clear, but seem quite likely.

7
Metabolism Versus Stimulation

Interaction of PAF with macrophages may have additional effects because of the ability of these cells to produce PAF acetylhydrolase. The product of this enzymatic activity is LPC (with either an alkyl or acyl *sn1* substituent depending on the type of PAF). This could occur within the cell or in the local extracellular environment. While such inter-conversion between these phospholipid mediators has not received much attention, transcellular metabolism and biosynthesis has been well documented for eicosanoids. The process adds complexity to our overall understanding of lipid mediators.

7.1
Transcellular Metabolism and Biosynthesis

The terms transcellular metabolism and biosynthesis are used to describe the production of intermediates in the eicosanoid, PAF, or lysophosphatide pathways by one cell, followed by their secretion or release to the outside, their subsequent uptake by other cells in the environment for further metabolism to mature mediators, which then are themselves secreted from the secondary cells for subsequent action (by ligation of specific receptors) on nearby cells.

The best example of this phenomenon is the synthesis of LTA_4 from arachidonate by 5-lipoxygenase (5-LO) on the nuclear membrane in cells such as neutrophils that do not contain the appropriate LTC_4 synthase to complete the synthesis of sulfidopeptide leukotrienes. In the presence of platelets, endothelial cells, or macrophages which do have this latter enzyme, the LTA_4 is efficiently taken up, metabolized to LTC_4 (Maclouf et al. 1996; Fradin et al. 1989; Maclouf et al. 1989; Sala et al. 2000) and then secreted into the environment to act on cells with appropriate receptors for these sulfidopeptide leukotrienes, including the macrophages themselves (Grimminger et al. 1991; Fukai et al. 1996).

Activation of macrophages and other inflammatory cells has been shown not only to result in synthesis and release of PAF, but in most cases where this is carefully examined, of LPC, and/or of lyso-PAF (i.e., alkyl-lyso-PC) as well. Uptake of these [possibly by scrambling, possibly via receptor engagement (Ohshima et al. 2002)] is rapid and efficient and can result in esterification by

the PAF acetyltransferase, i.e., the synthesis in this secondary cell of PAF or reacylation with longer chain fatty acids to add to the PC pool (Ohshima et al. 2002). Since macrophages contain and secrete high levels of PAF acetylhydrolase, any PAF they synthesize will always be subject to hydrolysis, reuptake, and re-esterification. The final balance of products is therefore likely to be highly dynamic, depending significantly not only on the initial production of lyso-PAF, the availability of acetyl-CoA, and levels of active acetyltransferase, but also on the extracellular environment. Although not much studied to date, similar effects may be seen with the lysophosphatides

8 Summary

This chapter has addressed a number of elements in the pharmacology, biochemistry, and biology of extracellular phospholipid mediators. It has focused on special features of phospholipids as mediators including mechanisms of secretion, membrane association, and presentation to their receptors. A number of similarities between the different groups of phospholipids were emphasized, even though the lysophosphatides and PAFs act through seven transmembrane, G protein-linked receptors and PS does not. One of the main points perhaps is that because of these special features, the pharmacology may be significantly more complex. On the other hand, these molecules are highly active, which provides real motive for appropriate pharmacological manipulation probably most easily at the level of the specific receptors. The phospholipid mediators discussed herein have very broad biologic activities. Some of this may be because of the numerous receptors (e.g., the EDG family). With others, the breadth cannot be accounted for in receptor heterogeneity (e.g., PAF) and more likely lies at the level of cell response variation and or multiple signaling pathways. Overall, with the possible exception of the PS receptor (study of which itself is in its infancy) the macrophage has not been a major focus for investigation of either these mediators' production or responses. This should be rectified.

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Part 3
Modulation of Specialised Macrophage Activities

Dendritic Cells Versus Macrophages as Antigen-Presenting Cells: Common and Unique Features

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Abstract Dendritic cells (DC) and macrophages contribute to both the innate and adaptive immune responses. It is becoming clear that DC and macrophages can be derived from common precursors, and that monocytes differentiate into DC under defined experimental conditions. Multiple types of DC and macrophages exist with different functional roles. Both immature DC and macrophages have significant phagocytic ability and are recruited by chemokines and cytokines to inflammatory sites. Upon encountering antigen or inflammatory stimuli, DC and macrophages become activated and responsible for several distinct non-specific and specific immunological functions. Most importantly, different stimuli, i.e. different pathogen-associated molecular patterns trigger different DC outcomes. Thus, the different DC subsets regulate the processing/delivery of antigen and provide a variety of costimulatory surface molecules, soluble cytokines and chemokines. DC are uniquely capable of activating primary immunity. This has driven the use of DC for tumour immunotherapy.

Keywords Dendritic cells, Macrophages, Differentiation, Sentinel function, Primary immunity, Immunotherapy

1 Introduction

Dendritic cells (DC) and macrophages belong to a family of antigen-presenting cells (APC). DC show certain similarities with macrophages, such as their differentiation pathway and sentinel functions but also have unique properties, such as efficient induction and maintenance of primary immune responses. Whilst the differentiation pathway that generates human DC in vivo remains unknown, DC and macrophages can be generated from common precursors under defined experimental conditions. As efficient sentinels, DC, like macrophages, are capable of recognizing danger signals derived from pathogens, taking up, processing and presenting antigen. However, DC differ from macrophages in key functions, notably the processing and delivery of antigen and the provision of costimulatory and accessory signals. These unique properties enable DC to function as effective APC, uniquely able to initiate primary immune responses and regulate adaptive immune responses. Because of their capacity to induce and maintain primary immune responses, DC are attractive vehicles for tumour immunotherapy.

This review points out similarities between DC and macrophages in their differentiation pathways and sentinel functions, as well as unique features of DC such as initiation and regulation of primary immune responses. Much of this work depends on the ability to discriminate DC populations with monoclonal antibodies. The currently limited but increasingly important field of DC differentiation antigens has been reviewed elsewhere (Hart et al. 2001, 2002).

2 Differentiation Pathways of DC and Their Relation to Macrophages

Human DC are found in almost all organs and represent a heterogeneous cell population. Based on phenotype and current views as to DC development, the different DC subsets are now universally subdivided into a minimum of two subsets. The myeloid-derived DC may include further subsets, but these certainly include the archetypical DC originally identified in mouse spleen. The lymphoid, plasmacytoid or confusingly, even the monocytoid DC is considered a very distinct subset, distinguished to a certain extent by its morphology but most effectively by its type I interferon (IFN)-producing capability. Myeloid-derived DC include Langerhans cells (LC), interstitial DC (e.g. dermal DC) and $CD11c^+$ DC isolated from lymphoid tissues. Their precursors are included in the $CD11c^+$ $CD123^{dim}$ blood DC subset. In addition, DC derived from monocytes (Mo-DC) are also considered as myeloid-derived DC. Plasmacytoid blood $CD123^{hi}CD11c^-$ DC, are the most probable candidates of lymphoid-derived DC and they are identified in thymus and other lymphoid organs. Their entry into the latter via high endothelial venules is a key distinguishing feature from myeloid DC, which enter lymph nodes via the afferent lymphatics.

Some subsets of myeloid-derived DC, dermal DC, LC and Mo-DC share common precursors with macrophages. $CD34^+CD1a^-$ precursors could generate DC when cultured with interleukin (IL)-7, tumour necrosis factor (TNF)-α, stem cell factor (SCF) and FLT3L. These precursors could also generate macrophages when cultured with macrophage colony-stimulating factor (M-CSF) (Dalloul et al. 1999). $CD34^+$ precursor cells obtained from cord blood or bone marrow differentiate into DC when cultured with granulocyte-macrophage (GM)-CSF and TNF-α (Caux et al. 1996; Caux et al. 1997). In this culture system, differentiation appears to occur via two independent, immature DC intermediates, defined by their exclusive expression of CD14 and CD1a. When cultured with GM-CSF and TNF-α, $CD14^+CD1a^-$ intermediates generate E-cadherin-mature DC, with a dermal or lymphoid-organ DC phenotype. They could also generate macrophages, when cultured with M-CSF. In contrast, $CD14^-CD1a^+$ intermediates generate $E\text{-cadherin}^+langerin^+$ LC-like DC. Differentiation of LC can also be achieved from the $CD14^+CD1a^-$ intermediates, in culture with transforming growth factor (TGF)-β (Jaksits et al. 1999). Similarly, LC-like DC can be generated by culturing monocytes (Geissmann et al. 1998) or blood $CD11c^+CD1a^+DC$ with GM-CSF, IL-4 and TGF-β (Ito et al. 1999).

Peripheral blood monocytes cultured with the cytokine combination of GM-CSF and IL-4 (Sallusto and Lanzavecchia 1994) or GM-CSF and IL-13 (Piemonti et al. 1995; Allavena et al. 1998) differentiate into immature Mo-DC. Differentiation of monocytes into DC occurs during the transendothelial migration of monocytes (Randolph et al. 1998) and takes place in the lymph nodes (Randolph et al. 1999). Interestingly, mouse monocytes, which migrate in vivo, differentiate into DC-like cells that have high late bead uptake capacity but lack CD11c (Randolph et al. 1999), the key DC subset marker. It appears that the minority $CD16^+$ monocyte subset may be at least partially committed to DC differentiation (Randolph et al. 2002). Peripheral blood monocytes can be made to differentiate into macrophages, when cultured with M-CSF or GM-CSF (Clark and Kamen 1987; Metcalf 1989).

Cytokines and undefined serum component(s) control the balance between the differentiation of monocytes into DC and into macrophages. The cytokine IL-6 exerts inhibitory effects on DC development and promotes differentiation of monocytes to macrophages after addition to GM-CSF and IL-4 cultures (Mitani et al. 2000). This action of IL-6 can be abrogated by TNF-α, lipopolysaccharide (LPS), IL-1β, CD40L and TGF-β1. Furthermore, certain immunosuppressive cytokines, such as IL-10 prevent the differentiation of monocytes into DC but not into macrophages (Allavena et al. 1998). The results obtained from serum-containing and serum-free culture experiments show that the humoral factor(s) in serum promote differentiation of monocytes into macrophages rather than into DC, when cultured with GM-CSF and IL-4 (Cao et al. 2000).

Notch receptors are conserved transmembrane receptors, which play a central role in regulating cell decision of bipotent precursors. Notch receptors expressed by bipotential progenitors are activated by neighbouring cells bearing Notch ligands, leading to differentiation of Notch-expressing cells along a lin-

eage-specific pathway. Interaction between the transmembrane receptor Notch and its ligand Delta-1 balance the differentiation of blood monocytes towards DC but not towards macrophages (Ohishi et al. 2001).

Immature Mo-DC exposed to TNF-α, LPS, CD40L or prostaglandin E2 (PGE2), following culture with GM-CSF and IL-4, acquire a final commitment towards mature DC expressing a high level of MHC class I and II, the costimulatory molecules CD80 and CD86, and the DC differentiation/activation molecules CMRF-44 (Vuckovic et al. 1998), CMRF56 (Hock et al. 1999) and CD83 (Zhou and Tedder 1996). In addition, type I IFN also promotes differentiation of monocyte to mature DC in culture with granulocyte (G)-CSF or with GM-CSF and IL-4 (Santini et al. 2000; Huang et al. 2001).

At least some subsets of myeloid-derived DC appear capable of differentiating into macrophages, again indicating a developmental link between DC and macrophages. Immature Mo-DC derived after culture with GM-CSF and IL-4 have limited DC commitment and acquire macrophage features after removing cytokines, unless stimulated to differentiate to mature Mo-DC (Palucka et al. 1998). A subset of DC found in the blood, which has a low expression of CD11c molecules, acquired macrophage features following exposure to M-CSF (Robinson et al. 1999). Curiously, the plasmacytoid blood $CD123^{hi}$ DC appear to have $CD14^+$ $CD16^+$ precursors (Ho et al. 2002).

Differentiation of DC from either $CD34^+$ precursor cells or peripheral blood monocytes and the developmental link between DC and macrophages observed in vitro requires in vivo confirmation that they represent physiological counterparts. Further research will address physiological mechanisms governing the differentiation of DC and the link between DC and macrophages. We have previously suggested that the preformed surveillance DC provide primary immune activation (Vuckovic et al. 1998). Recruitment of monocyte precursors and differentiation into DC may represent an inflammatory boost pathway for antigen presentation. Experiments can be designed to address this hypothesis.

3 DC and Macrophages in Pathogen Recognition

DC and macrophages function as sentinels for the cognate and innate immune systems. In the peripheral tissues, DC are found in an immature form characterized by their ability to recognize pathogen-associated molecular patterns (PAMP) shared by large groups of pathogens. Immature DC recognize PAMP through phylogenetically conserved Toll-like receptor (TLR) family members. In humans, six TLR homologues have been reported (Rock et al. 1998) and at least four others have been identified (Bowie and O'Neill 2000). All are type I integral membrane receptors with extracellular leucine-rich repeats and a cytoplasmic portion that is homologous to the signalling domain of the IL-1R. In mice, gene knockout studies indicate that TLR2 is required for gram-positive responses such as peptidoglycan (PGN) (Takeuchi et al. 1999) and outer membrane protein A (OmpA) (Jeannin et al. 2000), and TLR4 is essential for gram-

negative responses, including bacterial toxin LPS (Poltorak et al. 1998). TLR3 recognizes double-stranded RNA (dsRNA) or a synthetic dsRNA analogue poly(inosinic acid)–poly(cytidylic acid) [poly (I:C)], a molecular pattern associated with viral infection (Alexopoulou et al. 2001). TLR9 mediates immune responses to unmethylated CpG dinucleotides in the bacterial DNA (Hemmi et al. 2000). The function of other TLRs has yet to be defined.

Whilst several TLR are found on DC, the TLR are more abundantly expressed on macrophages. Mo-DC express TLR1, 2, 3 and 4 whereas TLR5 is barely detectable (Kadowaki et al. 2001; Visintin et al. 2001). Plasmacytoid blood $CD123^{hi}$ DC express TLR7 and 9 (Kadowaki et al. 2001). Macrophages express an abundance of TLR including TLR1, 2, 4, 5 and 8 (Visintin et al. 2001). It is of interest that TLR2 and 4 are required for responses to a number of PAMP and are expressed on both immature Mo-DC and macrophages, which perhaps reflects their similar functions. In accordance with TLR expression patterns, Mo-DC respond to the TLR2-ligand PGN, and plasmacytoid $CD123^{hi}$ blood DC respond to the TLR9-ligand unmethylated CpG dinucleotides in bacterial DNA (Hartmann et al. 1999; Kadowaki et al. 2001). Macrophages respond to microbial molecules known to trigger signalling via TLR2, TLR3 and TLR4, such as PGN, poly (I:C) and LPS, respectively.

Signalling through TLR drives DC and macrophages to produce proinflammatory cytokines. Signalling through TLR2 stimulates Mo-DC to produce large amounts of TNF-α in response to PGN. In contrast, signalling through TLR9 stimulates plasmacytoid blood $CD123^{hi}$ DC to produce IFN-α/β, during antibacterial immune responses. Signalling through TLR2, TLR3 and TLR4 empowers macrophages with the ability to produce large amounts of TNF-α and IL-6 during gram-positive or gram-negative immune responses (Kadowaki et al. 2001).

Recognition of PAMP drives the maturation of DC and progressive downregulation of TLR. This coincides with a functional switch from sentinel to antigen-presenting function. Mature Mo-DC lack any TLR. This results in a loss of responsiveness to LPS and a loss of TNF-α-producing capability. Upon maturation, plasmacytoid blood $CD123^{hi}$ DC downregulate expression of TLR7 and TLR9, lose their ability to produce IFN-α/β and acquire the ability to present antigen to T cells (Kadowaki et al. 2001; Visintin et al. 2001).

The presence of only a limited set of TLR on DC suggests that they might have a restricted ability to recognize PAMP and subsequently have lesser functional plasticity in response to pathogens, compared to macrophages. The recognition of PAMP by innate receptors on DC probably provides stress signals required for antigen targeting and cross-presentation by DC. The recognition of PAMP by macrophages enhances the elimination of bacteria. The possibility of cellular cooperation, in which macrophages provide antigen and relevant signals to DC was raised some time ago (McKenzie et al. 1989).

4
DC and Macrophages Differ in Their Ability to Cross-Present Exogenous Antigens

Initial investigations of DC and macrophages focused on the uptake of exogenous antigens for processing and presentation in the context of MHC class II molecules to $CD4^+$T lymphocytes.

The generation of cytotoxic effector T lymphocytes (CTL) responses to tumours (Berard et al. 2000), viruses (Sigal et al. 1999), bacterial antigens (Lenz et al. 2000), graft tissue (Bevan 1976) and even self-antigens (Kurts et al. 1996) also requires the presentation of exogenous antigen by MHC class I molecules on the surface of APC, a process termed cross-priming or cross-presentation. Both Mo-DC and macrophages can take up exogenous antigens in the form of soluble proteins, particulate antigen and cell-associated antigens derived from apoptotic or necrotic cells, and then process and cross-present them in the context of the MHC class I molecules (Rock et al. 1993; Shen et al. 1997). Other reports have indicated that cross-presentation is a specific property of DC (Mitchell et al. 1998; Rodriguez et al. 1999).

DC and macrophages use scavenger receptor-mediated endocytosis to take up exogenous antigen derived from apoptotic cells. Mo-DC use $\alpha_v\beta5$ integrin in cooperation with CD36 and thrombospondin to form a molecular bridge to their apoptotic target (Albert et al. 1998). Macrophages use $\alpha_v\beta3$ integrin, CD36, thrombospondin and the phosphatidylserine-binding protein, the cognate receptor for externalized phosphatidylserine on apoptotic cells (Savill et al. 1992; Fadok et al. 2000). Uptake of soluble proteins by phagocytosis or macropinocytosis (Kovacsovics-Bankowski et al. 1993; Norbury et al. 1995) also leads to cross-presentation but requires higher antigen concentration and could be less relevant in vivo than receptor-mediated endocytosis.

DC and macrophages also express an extensive range of both type I and type II C type lectins that appear to have a role in antigen uptake. The lectin molecules DEC-205 (CD205), MMR (CD206), DC-SIGN (CD209) and BDCA-2 are differentially expressed on DC, Mo-DC and macrophages (Kato et al. 2000; Osugi et al. 2002). DEC-205 delivers antigen deep into the endocytic pathway. Other lectins such as DC-SIGN (Geijtenbeek et al. 2000) and BDCA-2 (Dzionek et al. 2001) are internalized, resulting in effective antigen processing.

Mo-DC are able to prime naïve T cells, and induce CTL responses to antigen derived from apoptotic cells and clear pathogens responsible for the induction of apoptotic cell death (Albert et al. 1998). Macrophages are more efficient at taking up antigens derived from apoptotic cells than Mo-DC, but they degrade rather than cross-present the ingested antigens and subsequently suppress inflammatory responses (Voll et al. 1997; Fadok et al. 1998). This could be explained by the direct transport of internalized antigens from endosome to the cytosol, which exists in DC but not in macrophages (Rodriguez et al. 1999). Direct entry of exogenous antigen into the cytosol results in antigen introduction into the classical transporter associated with the antigen processing (TAP)-de-

pendent MHC class I presentation pathway. Noncytosolic pathways are related to endocytic processing by macrophages and involve loading of peptides on post-Golgi MHC class I molecules (Yewdell et al. 1999).

Exogenous antigens chaperoned by heat shock proteins (HSP) are released into the extracellular milieu during necrotic cell death. A wide array of antigenic peptides are chaperoned by HSP such as tumour-antigenic peptides (Ishii et al. 1999), viral epitopes (Greenstone et al. 1998) or corresponding epitopes from ovalbumin or β-galactosidase-transfected cells (Arnold et al. 1995). They are chaperoned by different HSP including gp96, hsp90 and hsp70. DC and macrophages use the HSP receptor, CD91 to take up HSP-peptide complexes (Binder et al. 2000; Basu et al. 2001). Interactions of HSP with CD91 induce expression of costimulatory molecules on the DC and stimulate both DC and macrophages to secrete cytokines such as TNF-α, GM-CSF and IL-12.

Both DC and macrophages are able to cross-present HSP–peptide complexes in the context of MHC class I molecules and induce antigen-specific CTL responses. Presentation of HSP–peptide complexes occurs exclusively in tissue but not in blood as a result of localized necrotic cell death. Extremely small quantities of peptides (nanograms or picograms) are sufficient to induce CTL responses. Peptides alone or chaperoned by non-HSP proteins such as albumin do not induce CTL responses (Anderson and Srivastava 2000).

Demonstration in vitro that a cell can cross-present antigen does not prove that cell is responsible for the special function of cross-presentation in vivo. There is the paucity of evidence concerning the identity of APC involved in the latter process. den Hann and colleagues identified, for the first time, the APC in lymphoid tissue involved in cross-presentation (den Haan et al. 2000) and showed that mouse $CD8^+$ DC but not $CD8^-$ DC, cross-present antigen in the spleen. The number of cross-presenting APC appears to be very low, and only 1% of these cross-present cell-associated antigen to $CD8^+$ T cells. A similarly low percentage of activated DC is found in human lymphoid tissue (Summers et al. 2000). Rat $CD4^-$ DC containing apoptotic cell remnants have been found to migrate to the T-cell areas of lymph nodes (Huang et al. 2000). These rat $CD4^-$ DC may be the rat equivalent of the mouse $CD8^+$ DC and may be involved in cross-presentation. Macrophage-like cells with cross-presenting function have been isolated from tumours (Ostrand-Rosenberg et al. 1999). The role of distinct types of DC and macrophages in cross-presentation in vivo needs further investigation.

5 The Differentiation of DC

The differentiation (maturation) of DC reduces the high rate of antigen uptake, increases the secretion of the cytokines and chemokines needed for the migration of DC and induces the expression of the antigen presenting and costimulatory molecules required to enhance antigen presentation and initiate an immune response. Oligonucleotide array and proteomics studies indicate the pro-

grammed expression of many genes during DC differentiation, most of which are likely to enable the differentiating and migrating DC to respond to microenvironmental regulatory signals.

Most studies show the changes in gene and protein expression that occur during differentiation/maturation of immature Mo-DC into mature Mo-DC (Dietz et al. 2000; Le Naour et al. 2001). This differentiation is accompanied by changes in the expression of several genes encoding proteins involved in cell adhesion and motility. The adhesion molecules, galectin 2, galectin 3, CD11a/LFA-1α, ninjurin 1, macmarcks, syndecan 2, CD44E and presenilin 1, are all downregulated. A truncated form of Cadherin-8 is downregulated. Differentiation of DC induces a switch from galectin 3 (high in immature DC) to galectin 9 (high in mature DC). Expression of secreted proteins involved in cell mortality, autotaxin-t and semaphoring E, macrophage capping protein and vimentin, are upregulated. The concomitant decrease in expression of integrins and other cell adhesion molecules plus the increase in expression of genes involved in cell motility almost certainly contributes to the enhanced migration properties of mature DC compared with immature DC (Barratt-Boyes et al. 2000).

The differentiation of DC is accompanied by marked changes in the expression of cytokines and chemokine expression as well as their receptors. Several genes encoding proinflammatory cytokines and their receptors, such as prointerleukin 1β, TNF-α, CD163, C5a anaphylatoxin receptor, IL-6R, and TNFR, are downregulated. The chemokines, which act as potent neutrophil chemoattractants and activators including CTAPIII, MIP2-α, MIP-2β, ENA78, PF4 and IL-8, are downregulated. Genes encoding anti-inflammatory proteins such as cyclophilin C and TSG-6, are upregulated. The differentiation of DC is also accompanied by the upregulation of osteopontin, a key cytokine involved in T-cell activation (Ashkar et al. 2000). Mac-2-binding protein, an adhesion molecule involved in natural killer (NK) and lymphokine-activated killer (LAK) cell activation and secretion of IL-2 (Ullrich et al. 1994), is upregulated. Upregulation of TGF-α is also observed during DC differentiation. Among leukocytes, only activated macrophages secrete TGF-α. TGF-α secreted by DC may participate in wound healing and repair (Schultz et al. 1991), tumourigenesis (DiGiovanni et al. 1994) and/or providing support for DC homing.

Differentiated DC express IL-7, IL-15 and their appropriate ligands, which stimulate T-cell expansion (Dietz et al. 2000). Such expression of cytokines and their cognate ligands may be analogous to the expression by differentiated DC of IL-12 together with IL-12R, another potent T-cell stimulus (Grohmann et al. 1998). Differentiation of DC is accompanied by increasing levels of CCR7, TARC and STCP-1, all of which are involved in chemotaxis and are needed to target cells into the lymph nodes. Another gene transcribed selectively during DC differentiation is indoleamine 2,3-deoxygenase (IDO). IDO degrades tryptophan required for T-cell proliferation and subsequently suppresses T-cell proliferation (Mellor and Munn 1999). DC that transcribe IDO can reduce local levels of available tryptophan by the action of IDO and protect themselves from the activated cytotoxic T cells they stimulate. The identification of another putative en-

zyme DCAL (Dekker et al. 2002) induced during DC differentiation indicates there is much more to be learnt about these processes.

Several HSP that participate in antigen processing and presentation, hsp73, hsp27, and calreticulin are also regulated during DC differentiation. Hsp73 binds specifically to the cell surface of monocytes and DC lines, is internalized spontaneously by receptor-mediated endocytosis (Arnold-Schild et al. 1999) and is upregulated during DC differentiation. The role of hsp27 upregulation during DC differentiation is less clear. Increased hsp27 promotes resistance of monocytes to apoptotic cell death (Samali and Cotter 1996). In contrast to hsp73 and hsp27, calreticulin is downregulated during DC differentiation due to post-translational modification. Calreticulin participates in the assembly of MHC class I with peptide and β2-microglobulin in the endoplasmic reticulum, a process required for the presentation of antigenic peptides to cytotoxic T lymphocytes at the cell surface (Krause and Michalak 1997). Proteomic analysis of DC identified a truncated form of calreticulin 32 present only in DC (Le Naour et al. 2001). This form contains the P-domain, a site of chaperone activity, the C-domain, which contains the endoplasmic reticulum retrieval sequence, but lacks the N-domain. The function of calreticulin 32 in mature DC is under investigation.

6
DC Regulate the Adaptive Immune Response

Mature DC provide a permissive environment for inducing immune responses. Both myeloid DC and plasmacytoid CD123^{hi} DC can induce T helper (Th)1 and Th2 immune responses, and despite initial suggestions, there is no stable DC phenotype or subset distinction, which polarizes distinct, Th1 or Th2 immune responses. The type and magnitude of Th immune responses is dependent on the differentiation/activation status of DC regulated by the type of differentiating stimulus, duration of DC activation and the DC–T cell ratio.

Following exposure to CD40L, LPS (Vieira et al. 2000) or dsRNA (Verdijk et al. 1999), mature DC produce IL-12 and consequently drive Th1 responses. Blood myeloid CD11c^{+} DC can generate higher numbers of Th1 effector cells than Mo-DC obtained from the same donors (Osugi et al. 2002). In contrast, PGE2 promotes differentiation of mature DC that produce low levels of IL-12 and drive Th2 immune responses (Kalinski et al. 1998). Type I IFN could promote mature DC with the ability to induce Th1 or Th2 responses, depending on the cytokine combined with type I IFN. Mature DC derived in culture with type I IFN and GM-CSF produce IL-15 and promote Th1 immune responses (Santini et al. 2000). In contrast, mature DC derived in culture with type I IFN, GM-CSF and IL-4, produce IL-10 and favour Th2 immune responses (Huang et al. 2001). Yssel's medium supplemented with LPS or IFN-γ promotes differentiation of mature DC that produce low levels of IL-12, increased levels of IL-10 and direct differentiation of Th cells towards the Th0/Th2 responses (Chang et al. 2000).

DC taken at early time points after induction of maturation induce Th1 responses. DC that are "exhausted" or "polarized" as a results of prolonged activation, lose the ability to produce IL-12 and induce Th2 responses (Langenkamp et al. 2000). The DC:T-cell ratio in these experiments influences outcome, e.g. a high DC:T-cell ratio promotes Th1 and a low DC:T-cell ratio promotes Th2 responses (Tanaka et al. 2000).

Human plasmacytoid $CD123^{hi}$ DC could induce Th1 or Th2 immune responses depending on the type of differentiation factors. When cultured with IL-3, they preferentially promote Th2 immune responses, whereas activated with viruses they prime naïve T cells to produce IFN-γ and IL-10 (Cella et al. 2000; Kadowaki et al. 2000).

Effector $CD4^+$ T cells induced by mature DC are required for recruitment of other effectors such as macrophages and eosinophils and for the induction of $CD8^+$ T-cell mediated CTL responses to cross-presented exogenous antigens. In addition to T-cell activation, it is important to note that cross-presentation can also induce T-cell tolerance. In lymph nodes, in the absence of effector $CD4^+$ T cells, memory $CD8^+$ T cells divided and were subsequently deleted leading to tolerance. In contrast, in the presence of $CD4^+$ T cells, effector IFN-γ-producing CTL occurred (Albert et al. 2001). The contribution of $CD4^+$ T cells can be replaced by CD40 crosslinking and inflammatory cytokines. Macrophages are not capable of generating IFN-γ-producing CTL even in the presence of CD40 crosslinking (Albert et al. 1998) and T cells exposed in this way remain immunologically ignorant. Endogenous antigen-loading via a classical MHC class I pathway allows both macrophages and DC to trigger production of IFN-γ-producing CTL.

7 New Approaches in DC Immunotherapy

Because of their ability to cross-present exogenous antigen and induce and maintain efficient primary immune responses, DC are the main cellular vehicle for clinical trials of vaccine strategies aimed to initiate CTL responses to tumours and pathogens (www.mmri.mater.org.au). In this context, macrophages are not attractive candidates for use in immunotherapy because of their failure to induce primary immune responses.

Despite the variety of strategies that have induced tumour-specific immune responses, the optimal DC-based strategies for human trials still remain to be determined. The most commonly used, clinically approved, approach is based on loading empty MHC class I molecules on DC with exogenous peptides. However, this is limited by peptide restriction to a given HLA type, induction of CTL responses only and limited patient responses to defined tumour antigen. Indeed, many DC-based immunotherapy protocols in human cancer have shown limited efficacy (Nestle et al. 2001), challenging research for improved strategy. Which type of DC preparation to use and how to administer it remain major issues.

As an alternative, apoptotic or necrotic tumour cells can be provided to DC for cross-presentation of tumour antigen. The use of apoptotic or necrotic killed tumour cells as a source of tumour antigen should provide both MHC class I and class II epitopes, leading to diverse immune responses involving polyclonal CTL and helper $CD4^+$ T cells. Moreover, helper $CD4^+$ T cells are able to recruit other effectors such as macrophages and eosinophils. DC loaded with killed allogeneic melanoma cells are able to induce differentiation of naïve T cells into CTL that are specific for a broad spectrum of shared melanoma antigens (Berard et al. 2000). This demonstration of cross-priming against shared tumour antigens builds the basis for using allogeneic tumour cells lines to deliver tumour antigen to DC for vaccination protocols.

Emerging data suggest that HSP participate in antigen presentation and play a central role in the induction of primary immune responses by DC. Tumour-derived HSP are used to treat autologous tumours in patients with advanced tumours, renal cell carcinoma and metastasis melanoma (Anderson and Srivastava 2000). Results showed that the autologous gp96 vaccine was effective in the adjuvant setting and post-vaccination stabilization of disease and CTL-restricted responses against autologous tumours was demonstrated.

New approaches based on the use of apoptotic or necrotic allogeneic tumour cell lines or HSP prepared from autologous tumours to induce responses against tumour antigens may have the advantages of applicability to many patients regardless of HLA type, as well as the generation of tumour-specific $CD4^+$T responses, which may recruit other effectors such as macrophages and eosinophils. The possibility of administering these subcutaneously as adjuvant killed tumour cells or HSP may even avoid antigen loading or transfection of DC prepared in vitro. These approaches will need to be studied and contrasted with more defined methods of antigen loading DC, which are more attractive to the regulatory authorities.

In summary, we have much to learn about the relationship of DC subsets to macrophages and their potential cooperative interactions. The plethora of emerging molecular data give scientists new opportunities and that data will undoubtedly translate rapidly into clinical applications.

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The Osteoclast

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Abstract The osteoclast is the cell that resorbs bone. It has been known for many years that it is formed from cells of the mononuclear phagocyte system, and that its formation and function are governed by osteoblastic cells. Recently, the molecular basis for this regulation was identified: osteoblastic cells induce osteoclastic differentiation in immature mononuclear phagocytes through expression of macrophage colony-stimulating factor (M-CSF) and receptor-activator of NFκB ligand (RANKL). Osteoblastic regulation of bone resorption is assisted through secretion of an inhibitor, osteoprotegerin (OPG), a soluble (decoy) receptor for RANKL. Transforming growth factor beta (TGF-β), which is present in bone matrix in large amounts, is also essential for osteoclast formation, at least in vitro. Surprisingly, TNF-α can substitute for and is strongly synergistic with RANKL for osteoclast-induction. TNF-α is widely expressed, and RANKL can also be present in situations that are not associated with osteoclast formation, so that the presence of large quantities of TGF-β in bone matrix might explain why osteoclast formation is essentially confined to the bone microenvironment. In this review, recent data concerning the mechanisms underlying the induction of osteoclastic differentiation and function are described, together with recent findings concerning the mechanisms through which osteoclasts adhere to and resorb bone. Several of these mechanisms are currently being exploited for the development of novel therapies for diseases, such as osteoporosis, that are caused by excessive bone resorption.

Keywords Osteoclast, Osteoblast, RANKL, TNF-?, TGF-?, Bone

Abbreviations

$\alpha_v\beta3$	$\beta3$integrin alpha v beta 3
CTR	calcitonin receptor
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinase
OPG	osteoprotegerin
RANK	receptor activator for NF-κB
RANKL	ligand for RANK
TRAF	TNF receptor-associated factor
TRAP	tartrate-resistant acid phosphatase
TRANCE	tumour necrosis factor-related activation-induced cytokine

1 Introduction

Bone resorption is crucial to the normal development and maintenance of the skeleton, and for the regulation of plasma calcium levels. In development, deficient resorption leads to osteopetrosis and failure of tooth eruption. In the adult, the continuous physiological remodelling of bone, whereby aged or fatigued bone is removed and replaced by new, is dependent on bone resorption. Excessive bone resorption is the key pathophysiological event underlying several diseases, including malignant hypercalcaemia and postmenopausal osteoporosis, in humans.

It has long been the consensus that osteoclasts are responsible for bone resorption. Their origin, though, has until recently been hotly contested. Their multinuclear and debriding characteristics suggested an origin from macrophages, but kinetic and histodynamic studies argued that they are locally derived, and there was even a popular idea that they could interconvert with osteoblasts, the cells that form bone. There have been striking advances in the last few years that have established a very close relationship between these cells and macrophages, and have illuminated many of the mechanisms involved in their formation, regulation and function.

2 Mechanisms of Bone Resorption

The defining characteristic of the osteoclast is its ability to resorb bone. When osteoclasts are placed on a bone surface in vitro, they make deep excavations with extraordinary speed. Macrophages can digest ingested bone particles but only the osteoclast can dissolve bone by an extracellular mechanism; and it achieves this unaided by other cell types (Chambers et al. 1984a). Actively re-

sorbing osteoclasts establish a circle of close contact with the bone surface, known as the sealing zone, below a peripheral ring of cytoplasm from which organelles are excluded (the clear zone of electron microscopy). This clear zone corresponds to a ring of F-actin, which correlates well with functional activation. The bone-apposed surface of the osteoclast circumscribed within this sealing zone is thrown into deep folds to form the 'ruffled border' seen by electron and light microscopy. In this area protons and acid hydrolases are extruded onto the bone surface. The mineral (calcium hydroxyapatite) and organic (predominantly collagen) components of bone are thereby dissolved. Solubilisation of organic components might continue during transocytic vesicular transport of the released material to the basal surface of the osteoclast, which is usually in contact with a blood vessel (Halleen et al. 1999).

Osteoclasts were originally identified as multinuclear cells, but it is not known why they are multinuclear. In vitro and in vivo, they can remain mononuclear, especially when the density is low, yet resorb bone (Kaye 1984; Fuller and Chambers 1989; Prallet et al. 1992), so that multinuclearity is not essential for their function. Multinuclearity may help in regulation, or improve the efficiency with which they can resorb: the effort expended in maintaining an extracellular resorptive micro-environment will be inversely related to the area of the interface with bone.

Osteoclasts have been shown to secrete protons into the subosteoclastic attachment zone (Baron et al. 1985; Silver et al. 1988). This occurs by targeted secretion of hydrochloric acid, by an H^+-ATPase proton pump, through the ruffled border (Vaananen et al. 1990). Concomitant with the development of the ruffled border, the number of intracellular acid compartments promptly decreases as vesicles containing proton pumps are transported to the surface that becomes the ruffled border. This sequence does not occur in osteopetrotic oc/oc mice, which lack an osteoclast-specific component of the vacuolar proton pump (Nakamura et al. 1997; Brady et al. 1999; Li et al. 1999; Nakamura et al. 1999; Scimeca et al. 2000). Mutation of the same subunit is one of the causes of osteopetrosis in humans (Frattini et al. 2000). The recent finding that vacuolar H^+-ATPase at the ruffled border contains osteoclast-specific subunits has further encouraged development of resorption-inhibitors that inhibit the osteoclastic proton pump (Hernando et al. 1995; Van Hille et al. 1995; Li et al. 1996). Recent data suggest that protons are not laterally contained by the so-called sealing zone, but are neutralised by mineral before loss by lateral diffusion occurs (Stenbeck and Horton 2000).

Protons for the proton pump are produced by cytoplasmic carbonic anhydrase II (CAII). High levels are present in osteoclasts (Gay and Mueller 1974). Inhibition of the enzyme suppresses bone resorption by isolated osteoclasts in vitro (Hall et al. 1991) and inherited deficiency leads to osteopetrosis (Sly et al. 1983). Excess cytoplasmic bicarbonate is removed via the chloride–bicarbonate exchanger located in the basolateral membrane (Hall and Chambers 1989). Correspondingly, there are large numbers of chloride channels in the ruffled border, which allow a flow of chloride anions into the resorption lacuna to maintain

electroneutrality (Schlesinger et al. 1997). Loss of a component of this channel (ClC9) leads to osteopetrosis in mice and man (Kornak et al. 2001).

After dissolution of the mineral phase, osteoclastic enzymes destroy the organic bone matrix. This process is sensitive to inhibition by leupeptin and other inhibitors of cysteine proteinases (Delaisse et al. 1987; Fuller and Chambers 1995). The overwhelmingly predominant cysteine proteinase in osteoclasts is cathepsin K, while cathepsins B, L and S are rare (Tezuka et al. 1994; Drake et al. 1996; Kamiya et al. 1998; Ishibashi et al. 2001). The enzyme differs from other cathepsins in its ability to act at a relatively high pH (pH 6), which might facilitate its extracellular activity, and in its ability to cleave native collagen in the triple-helical region (Garnero et al. 1998). This collagenase activity, otherwise seen only in the collagenases of the matrix metalloproteinase family and neutrophil elastase, depends upon the presence of chondroitin 4-sulphate, a component of bone (Li et al. 2000b). Cathepsin K has been immunolocalised to resorption pits (Xia et al. 1999). Mutation of the gene for cathepsin K in humans and mice results in osteopetrosis (Gelb et al. 1996; Saftig et al. 1998). This suggests that cathepsin K is the dominant proteinase responsible for the digestion of bone, with little compensation by other cathepsins.

Ostoclasts also express high levels of MMP-9 (gelatinase B) (Reponen et al. 1994). However, deletion of the gene for MMP-9 causes only a transient disturbance of bone resorption (Vu et al. 1998). Since inhibition of MMPs has no discernible effect on bone resorption by isolated osteoclasts (Fuller and Chambers 1995), the enzyme seems likely to be involved in some other function in the osteoclast, such as migration, or mobilisation of matrix-associated growth factors (Vu et al. 1998).

3
Osteoclast Differentiation and Activation

It was established in the 1970s, by parabiosis, tissue grafting, and bone marrow transplants, that osteoclasts were of haematogenous rather that local origin (see Marks 1983 for review). The mononuclear phagocyte series seemed the most likely candidate as precursors because, like the osteoclast, these cells are specialised for debridement and can fuse to form multinuclear cells in the presence of extracellular foreign bodies. On the basis of this origin, two predictions were made concerning the regulation of bone resorption (Chambers 1980). First, an origin for osteoclasts from inherently wandering cells suggests that their localisation is governed by local bone cells such as osteoblasts and osteocytes; and second, that this control needs to include some form of protection for bone from phagocytic attack as a foreign body, since implantation of unprotected bone provokes this reaction.

It became apparent, though, that while osteoclasts share some immunological markers with macrophages (see Athanasou 1996), they are also distinctive (see Chambers 1989; Helfrich and Horton 1993). Osteoclasts lack many markers characteristic of macrophages (e.g. Fc, C3 receptors) and express very high lev-

els of tartrate-resistant acid phosphatase (TRAP) and 'vitronectin receptors' (integrin $\alpha_v\beta 3$); and express calcitonin receptors (CTR), which are absent from macrophages. Most distinctively, osteoclasts ex vivo excavate bone within hours, while macrophages show no excavation whatsoever, even on extended incubation on bone surfaces (Chambers and Horton 1984; Chambers et al. 1984a).

When osteoclasts were extracted and tested, it was found that they were indeed governed by osteoblastic cells: agents known to stimulate osteoclasts in intact bone stimulated isolated osteoclasts only in the presence of osteoblastic cells (Chambers 1982; Chambers 1985; Mcsheehy and Chambers 1986; Thomson et al. 1986; Thomson et al. 1987). Similarly, osteoclast differentiation was found to depend on factors from osteoblastic/bone marrow stromal cells (Takahashi et al. 1988; Hattersley and Chambers 1989), the expression of which is increased by resorptive hormones (Fuller and Chambers 1998; Liu et al. 1998; Matsuzaki et al. 1998; Takeda et al. 1999). This led to the view that the induction of bone resorption depended upon a primary interaction of resorptive stimuli with osteoblastic cells, which responded by expressing factor(s) that induced the differentiation and activation of osteoclasts (Chambers 1992; Suda et al. 1995).

One of the factors produced by osteoblastic cells that supports osteoclast formation is M-CSF. The evidence for this came from the discovery that osteopetrosis in the op/op mouse was caused by a stop codon in the gene for M-CSF (Wiktor-Jedrzejczak et al. 1990; Yoshida et al. 1990). M-CSF is nevertheless not sufficient for osteoclast formation in vitro: an additional, osteoblast-derived factor is also required, or macrophages form by default (Hattersley et al. 1991; Takahashi et al. 1991). Nor is M-CSF essential in vivo: osteoclasts are present in op/op mice, but in reduced number, and the osteopetrosis resolves after a few weeks, perhaps as the resorptive burden decreases. Resolution is accelerated by transgenic over-expression of Bcl-2 in mononuclear phagocytes (Lagasse and Weissman 1997). The main role of M-CSF in osteoclast biology appears to be to enhance the survival and proliferation of precursors, and the survival of mature cells. More recently, it has also been found to induce the expression of RANK, the receptor for the osteoclast-inductive ligand RANKL (see below) (Arai et al. 1999). Flt3 ligand (FL) also induces RANK, and might account for the partial redundancy of M-CSF in osteoclast formation (Lean et al. 2001).

A major consequence of the identification of the role of M-CSF in osteoclast biology was the conclusion that, despite its distinct phenotype, the osteoclast derives from the mononuclear phagocyte system. This was reinforced when mice deleted for *c-fos* were found to be osteopetrotic, with absence of osteoclasts. Without c-Fos, precursors form macrophages, either by default or through arrested development, despite an osteoclast-inductive environment (Grigoriadis et al. 1994).

The osteoblast-derived ligand responsible for osteoclast differentiation and activation was independently discovered by Snow Brand Milk Products and Amgen. Both initially found a soluble inhibitor of osteoclast formation [osteoprotegerin (OPG), a soluble receptor of the tumour necrosis factor (TNF) superfamily] (Simonet et al. 1997; Tsuda et al. 1997). They used this to identify the cognate

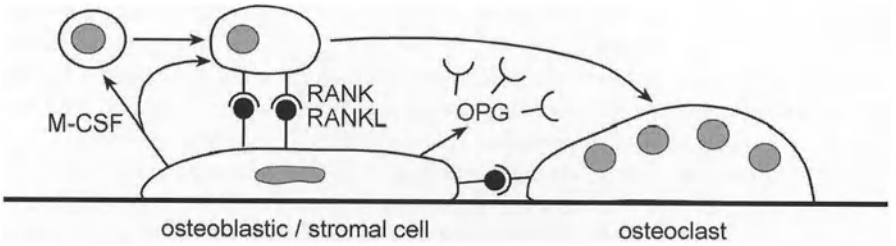

Fig. 1 Induction and regulation of osteoclasts by osteoblastic/stromal cells. Resorptive hormones such as parathyroid hormone, 1,25 dihydroxyvitamin D_3, interleukin-1 and TNF-α induce bone marrow stromal cells and osteoblastic cells to express M-CSF and RANKL. Haemopoietic cells are induced to express RANK by M-CSF or FL, and this interacts with RANKL on the surface of stromal cells to induce osteoclast formation and activity. The stromal cells also produce OPG, the soluble decoy receptor for RANKL. Expression of OPG is regulated in a manner reciprocal to RANKL

ligand, which was identical to the recently discovered TNF superfamily member TRANCE/RANKL (see Suda et al. 1999; Chambers 2000 for reviews).

RANKL is a type I transmembrane protein that (with M-CSF) replaces the need for osteoblastic/stromal cells in the induction and activation of osteoclasts from haemopoietic precursors in vitro (see Fig. 1). Expression of RANKL by osteoblastic cells is upregulated by agents that stimulate bone resorption. Mice in which the gene is deleted have osteopetrosis, caused by complete absence of osteoclasts. RANK is the receptor on osteoclasts and their precursors that interacts with RANKL. Soluble RANK and anti-RANK antibodies suppress osteoclast formation and activity, and deletion of the gene results in complete absence of osteoclasts (see Chambers 2000; Suda et al. 1999 for reviews).

RANK binds TNF receptor-associated factor (TRAF) 1,2,3,5 and 6 (Galibert et al. 1998; Kim et al. 1999). Animals double-mutant for p50/p52 nuclear factor (NF)-κB (Iotsova et al. 1997) or deficient in c-Fos, part of the activator protein (AP)-1 transcription factor complex, have osteopetrosis (Johnson et al. 1992; Wang et al. 1992). TRAF 2,5 and 6 have been shown to activate NF-κB downstream of TNF receptors (see Kim et al. 1999). The same TRAFs activate c-jun N-terminal kinase (JNK), which activates AP-1 (Kim et al. 1999). RANK activates not only NF-κB and JNK (Anderson et al. 1997; Wong et al. 1997; Darnay et al. 1998), but also expression of *c-fos* in osteoclasts (Matsuo et al. 2000). Deletion of the gene for TRAF6 causes osteopetrosis with normal numbers of non-resorptive osteoclasts (Lomaga et al. 1999), or absent osteoclasts (Naito et al. 1999). Many agents that do not induce osteoclast differentiation activate these signals, so RANKL presumably also induces unknown, osteoclast-specific signals.

Although precursors, whether from bone marrow, spleen, blood or peritoneum undergo osteoclastic differentiation when incubated in M-CSF and RANKL, macrophages also form. With continued incubation, only macrophages persist. In semi-solid media, osteoclasts always differentiate in colonies mixed with macrophages. This is unlikely to be the case on bone surfaces. It might be that

culture conditions, which are essentially pro-inflammatory (Thyberg 1996; Iyer et al. 1999) (see below) divert some of the precursors to macrophages; or the precursors that provide osteoclasts differ from those used in the above experiments; or there may be additional signals in vivo that ensure osteoclasts form. The proportion of cells that develops into osteoclasts is much greater if precursors are incubated on bone slices, rather than on plastic substrates (Fuller et al. 2000).

Transforming growth factor (TGF)-β is an essential cofactor for osteoclast formation, at least in vitro. It substantially increases the proportion of precursors that become osteoclasts (Sells Galvin et al. 1999; Fuller et al. 2000), and blockade of TGF-β signalling abolishes osteoclast formation, while increasing macrophage numbers (Fuller et al. 2000). Basal osteoclast formation by RANKL in vitro is likely to be due to TGF-β in serum and/or produced by the precursors themselves. In the osteoblast-containing cultures used to analyse osteoclasts before RANKL was discovered, TGF-β had been found to either stimulate or inhibit resorptive cells. Inhibition might reflect negative feedback, because TGF-β, which osteoclasts express, induces production of OPG, the soluble decoy receptor for RANKL, in osteoblastic cells (Murakami et al. 1998; Takai et al. 1998).

In common with many pro-resorptive agents, TNF-α induces osteoblastic cells to stimulate osteoclasts (Thomson et al. 1987), and accordingly induces RANKL expression in osteoblastic cells (Horwood et al. 1998; Hofbauer et al. 1999). Surprisingly (to those who had spent many years looking for such a factor) it also stimulates osteoclast formation and bone resorption in vitro through a direct action on osteoclasts and their precursors (Azuma et al. 2000; Kobayashi et al. 2000; Fuller et al. 2002). This RANKL-like action in vitro appears to be independent of but strongly synergistic with RANKL, and of similar potency (Fuller et al. 2002). However, the role of TNF-α in vivo is less clear: Mice deleted for TNF receptors have normal bone, unlike RANK-deficient animals; and while injection of RANKL cures osteopetrosis in RANKL deficiency, TNF-α does not cure osteopetrosis in RANK-deficient mice (Li et al. 2000a). Even in inflammation, osteoclast formation is dependent on RANKL: It is required for bone loss in experimental arthritis (Kong et al. 1999; Pettit et al. 2001). Osteolysis by TNF-α in vivo might occur through induction of RANKL in osteoblastic cells. Also, extremely low levels of TNF-α are strongly synergistic with RANKL in vitro. Thus, although TNF-α cannot substitute for RANKL, systemic or local TNF-α could promote both RANKL expression and responsiveness, and so increase bone resorption with minimal disturbance to the underlying, RANKL-mediated physiological patterns of osteoclastic resorption.

4 The Osteoclast and the Macrophage

The ability of TNF-α, a ubiquitous macrophage-activating agent, to induce osteoclasts from immature mononuclear phagocytes in vitro, raises several ques-

tions concerning the nature of the osteoclast, and its relationship with macrophages. A major effect of TNF-α on macrophages is the activation of bacteriocidal activity (NO/superoxide), which is primed by interferon (IFN)-γ and deactivated by TGF-β. TGF-β by itself enhances phagocytosis and lysosomal enzyme production, and it has been suggested that while IFN-γ induces cytocidal macrophages early in host defence, TGF-β diverts macrophage activity towards debridement in the subsequent healing phase (see Riches 1996; Letterio and Roberts 1998 for reviews). We found that for TNF-α, as for RANKL, TGF-β dramatically increases the proportion of precursors that become osteoclasts, and IFN-γ does the reverse (Fox et al. 2000). The signals through which TGF-β achieves this are unknown, but there is evidence that IFN-γ suppresses osteoclast formation through inhibition of TRAF6 (Takayanagi et al. 2000). Whatever the mechanism, the observations of Fox et al. (2000) imply that lineage in M-CSF-induced precursors is determined by TGF-β/IFN-γ, and merely activated by TNF-α. By analogy, the osteoclast is a lineage determined by TGF-β and activated by TNF-α/RANKL. This suggests a model in which the osteoclast is an alternative and equivalent destiny for macrophage precursors to that of the cytocidal macrophage; it is an activated variant of the debriding macrophage.

In vitro, the osteoclastic phenotype is induced by the particular combination of M-CSF, RANKL/TNF-α and TGF-β. However, while TNF-α is ubiquitous in vivo, osteoclast formation is essentially confined to bone. This might be because the pro-inflammatory cytokine TNF-α and the anti-inflammatory TGF-β do not co-exist at levels sufficient to induce osteoclastic differentiation. Even if they did, because TNF-α is pro-inflammatory, it will normally be accompanied by, or itself induce, inflammatory cytokines such as GM-CSF, IL-4, IL-12, IL-18 and IFN-γ. These are all potent anti-osteoclast agents (Hattersley and Chambers 1990; Lacey et al. 1995; Udagawa et al. 1997; Horwood et al. 2001; Miyamoto et al. 2001). The ability of these cytokines to inhibit osteoclast differentiation might be secondary to their role in macrophage induction/stimulation. In contrast, RANKL is not pro-inflammatory (Fox et al. 2000), and this might facilitate the specific activation of the debridement pathway in macrophages. It might be that RANKL, unlike TNF-α, cures osteoclast deficiency in mice deleted for RANKL because the former does not generate osteoclast-inhibitory cytokines.

This view of the osteoclast, as an activated, non-inflammatory, debriding macrophage, is consistent with the absence of associated inflammatory cells such as neutrophils at sites of resorption. It is noteworthy though that cells with many of the characteristics of osteoclasts, such as multinuclearity, high levels of TRAP and cathepsin K, can be seen in inflammatory sites, particularly where extracellular foreign material is present (Diaz et al. 2000; Buhling et al. 2001). The osteoclast might have evolved as a variant of such defence cells, which became professionalised for the extracellular destruction of a particular substrate of predictable composition.

This does not imply that all multinuclear cells are closely related to osteoclasts. Not only RANKL, but cytokines that strongly inhibit osteoclast formation, such as IFN-γ, IL-3, IL-4 and GM-CSF, induce macrophages to form multi-

nucleate giant cells in vitro. Even those foreign body giant cells that most resemble osteoclasts are probably distinct: If devitalised bone is implanted subcutaneously into mice, it soon becomes covered by multinucleate giant cells, but the bone is not resorbed (Chambers and Horton 1984; Popoff and Marks 1986). Resorption only occurs when osteoblasts appear. Macrophage giant cells cannot be induced to resorb bone by RANKL (Boissy et al. 2001). Multinuclear macrophages may well be as phenotypically diverse as mononuclear macrophages; multinuclearity occurs most readily in immature, 'responsive' macrophages (Möst et al. 1997) and may reflect the intensity of the stimulus or response, rather than its nature.

The osteoclast might then be seen as the equivalent of those specialised macrophage derivatives that populate other tissues under physiologic conditions, such as alveolar macrophages, Kupffer cells and microglia. The question arises, as it does for these cells, as to the extent to which osteoclasts derive from self-sustaining local precursors that were originally established from the blood, or derive continuously from the blood. For osteoclasts, this is completely unknown. If they are replenished from the blood, this does not seem to occur via a typical monocyte, because only a very small proportion (<3%) of monocytes can form osteoclasts in vitro (Quinn et al. 1994; Fuller and Chambers 1998). Osteoclast formation from blood cells also takes a surprisingly long time (>14 days in human), suggesting that origin is from a proliferative subpopulation, rather than from typical monocytes. The resistance of the majority of monocytes to osteoclast differentiation may reflect the resistance to osteoclast formation that develops in bone marrow cells during incubation in M-CSF (Arai et al. 1999; Wani et al. 1999).

Recent observations in a variant of Paget's disease suggest that osteoclasts derive from local precursors. At least some cases of this disease, which is characterised by foci of reckless osteoclasts, were found to be due to an inherited mutation that causes RANK to be overactive (Hughes et al. 2000). This implies that the foci of reckless osteoclasts derive from locally proliferative precursors that have developed a second, activating mutation (if the precursor were haematogenous, reckless osteoclasts would be systemic). Thus, it remains unknown whether in adulthood osteoclasts originate from typical monocytes, or from a subpopulation of immature peripheral blood mononuclear cells, or from a haematogenous cell that establishes a self-sustaining pool of precursors on bone surfaces. The distinction is critical to the design of in vitro models for recruitment mechanisms and precursor responsiveness.

5
How is the Spatial Control of Resorption Achieved?

A striking feature of bone cell biology is the complexity and dynamism of the patterns of osteoclastic localisation associated with bone morphogenesis and restructuring. These patterns depend on the ability of resident bone cells to direct

precursors and mature osteoclasts to the sites at which activation of bone resorption is appropriate.

Despite uncertainty regarding the nature of the precursor, some speculations are possible concerning localisation mechanisms. This might occur via direct tethering by M-CSF or RANKL, because both are expressed by bone cells as transmembrane forms. If so, they must be mutually redundant, because systemic administration of (soluble) M-CSF or RANKL to mice deficient in the respective genes for these proteins leads to cure of osteopetrosis with largely normal patterns of osteoclastic localisation. An alternative explanation for the ability of soluble RANKL to localise osteoclasts might be via TNF-α-like induction of adhesion receptors in endothelial cells, for RANKL specifically in bone endothelial cells. However, bone marrow transplants show that RANK is required only on osteoclasts and their precursors for localisation to occur (Li et al. 2000a). While the possibilities above have not been excluded, the most likely model for localisation is one in which bone cells generate patterns of adhesion molecules, such as integrin ligands [especially ligands for the alpha v beta 3 receptor ($\alpha_v\beta3$), which is highly expressed on osteoclasts], in addition to RANKL and M-CSF. An advantage of such a RANKL/M-CSF-independent localisation mechanism would be that it would enable the establishment of a pool of special, non-differentiated osteoclast precursors on bone surfaces.

A second, related question is: Once osteoclasts are localised, how is resorption induced? It might merely be a matter of increased expression of RANKL. RANKL clearly activates resorption behaviour by osteoclasts on bone or dentine slices (Fuller et al. 1998; Burgess et al. 1999). However, it seems unlikely that the same behaviour occurs on other substrates; this would be inappropriate and potentially destructive. It is more likely that there are recognition factors whereby bone allows or assists resorptive behaviour.

The role of the integrin $\alpha_v\beta3$ has received much attention in the context of osteoclast localisation and activation. $\alpha_v\beta3$ is highly expressed in osteoclasts and it is likely to play a role in migration, adhesion and perhaps endocytosis of resorption products (see Väänänen et al. 2000). Antibodies against $\alpha_v\beta3$, or RGD tripeptide-containing peptides such as echistatin and kistrin, are effective inhibitors of bone resorption in vitro and in vivo (Chambers et al. 1986; Horton et al. 1991; Lakkakorpi et al. 1991; Fisher et al. 1993), and resorption is reduced, although still present, in mice deleted for the β_3 gene (McHugh et al. 2000). Osteoclast localisation in vivo, however, appears unaffected by $\alpha_v\beta3$ blockade or deficiency (Masarachia et al. 1998; Yamamoto et al. 1998; McHugh et al. 2000). Surprisingly, $\alpha_v\beta3$ seems to be absent from the sealing zone of close attachment of the resorbing osteoclast to bone (Lakkakorpi et al. 1991; Masarachia et al. 1998), although actin ring formation, and probably migration of osteoclasts over the bone surface, depends on $\alpha_v\beta3$ ligation (Nakamura et al. 1999).

Although $\alpha_v\beta3$ is redundant for osteoclastic localisation, it does greatly assist bone resorption. However, the ubiquitous presence of $\alpha_v\beta3$ ligands makes it unlikely that signalling through this integrin alone is sufficient to induce resorptive behaviour. It seems very likely that there are alternative signals for localisa-

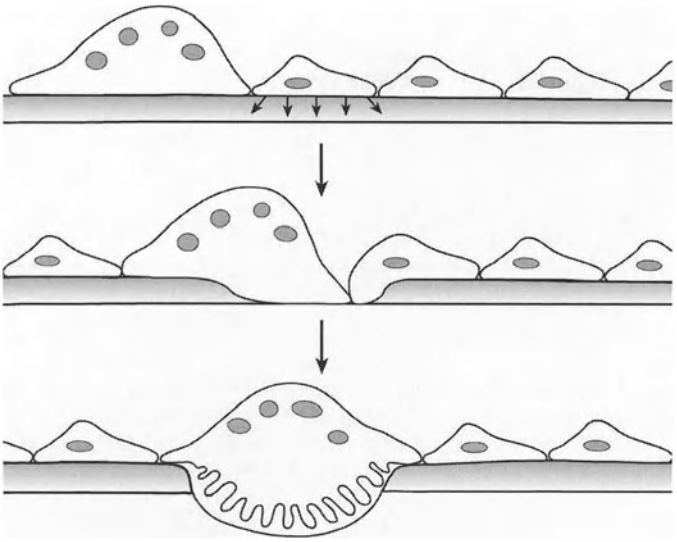

Fig. 2 A model for the mechanism of induction of bone resorption. Osteoclasts undertake resorptive behaviour only when in contact with bone mineral. Hormones and cytokines that stimulate bone resorption also stimulate osteoblastic cells to secrete interstitial collagenase. This exposes osteoclasts to bone mineral, which induces or enables resorptive behaviour. *Shaded area* represents non-mineralised organic material that covers bone surfaces. *Arrows* represent interstitial collagenase

tion, and probable that the substrate provides additional signals to the osteoclasts that resorptive behaviour is appropriate. Bone mineral might be involved in both processes: implantation of inorganic materials such as hydroxyapatite crystals into tissues evokes a foreign body giant cell (multinuclear macrophage) response that leads to sequestration and dissolution of the implanted material. Presumably, therefore, bone has evolved ways to protect itself from such phagocytic attack. One possibility is that the osteoblastic cells on bone surfaces retract to allow resorption (Rodan and Martin 1981). However, in experiments designed to test this idea, surface cells offered only minor protection against resorption (Karsdal et al. 2001). A second possibility is that the non-mineralised layer of organic material that lines bone surfaces represents the protection against phagocytic (or osteoclastic) attack. Then, when bone resorption is required, bone-lining cells remove the organic protective layer to expose bone mineral to 'phagocytic' recognition by the special debrider of bone, the osteoclast (Chambers 1980) (Fig. 2).

This model is consistent with a large body of evidence (see Chambers 1992; Fuller and Chambers 1995 for references). First, while osteoclasts cultured on bone slices require only cysteine proteinases to resorb the bone matrix, resorption in vivo depends upon both cysteine proteinases and interstitial collagenase. Second, interstitial collagenase expression is observed in bone-lining and osteocytic cells immediately adjacent to osteoclasts in bone tissue, but not in osteoclasts (Fuller and Chambers 1995; Zhao et al. 1999); and there is abundant evi-

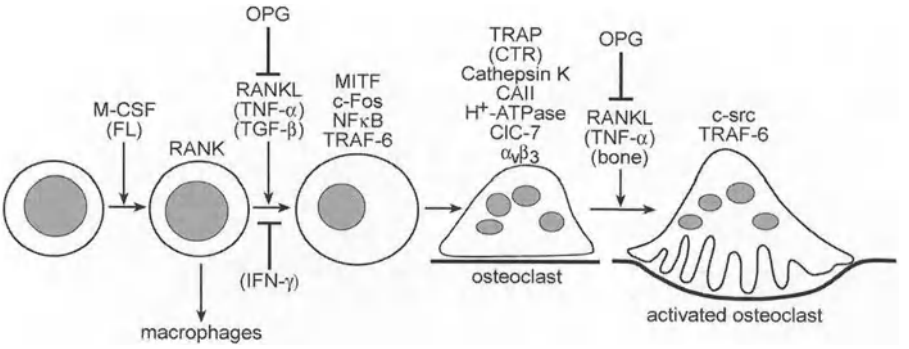

Fig. 3 Molecular mechanisms of osteoclast differentiation and function. An essential role in vivo for all the molecules, except those *in parentheses*, has been demonstrated by gene deletion/mutation. *CAII*, carbonic anhydrase; *CIC7*, chloride transporter; *CTR*, calcitonin receptor; *FL*, flt3 ligand; *MITF*, microphthalmia transcription factor; *TRAP*, tartrate-resistant acid phosphatase. See text for further details

dence that hormones that stimulate resorption strongly stimulate interstitial collagenase expression in osteoblasts (see Chambers 1992 for references). Third, osteoclasts readily resorb bone if mineral is exposed on the bone surface, but do not resorb bone that is unmineralised or has been demineralised (Chambers et al. 1984b; Chambers and Fuller 1985). Last, incubation of native bone surfaces with interstitial collagenase exposes mineral onto the surface, and renders the bone resorption-inductive for osteoclasts (Chambers et al. 1985). The observations provide compelling evidence for a model in which, while osteoclasts can undoubtedly digest all the components of bone including native collagen, unaided by other cell types, the induction of resorptive activity in osteoclasts depends on contact with bone mineral.

6
Pharmacologic Intervention in Bone Disease

Although it is merely the pawn, the osteoclast is invariably the agent, whenever there is excessive bone destruction. The commonest consequence is osteoporosis, but there are many other circumstances in which it is beneficial to suppress excessive bone resorption, such as in the bone destruction that accompanies metastatic and non-metastatic malignancy, rheumatoid arthritis, periodontitis and Paget's disease. The osteoclast has a very specialised function, compared to many other members of the macrophage family. This might be responsible for the wealth of molecules that are unique to or uniquely essential in these cells. The recent remarkable progress in identifying these molecules has provided many new potential targets for the specific and potent inhibition of osteoclastic function (Fig. 3). Many of these, and many other agents such as oestrogens, selective oestrogen receptor modulators (SERMS) and bisphosphonates, that specifically inhibit resorption through less-well understood mechanisms, and

many agents beyond, are used in current therapy, or are the subject of drug discovery and development programmes. A detailed treatment of this area is beyond the scope of this article, and the reader is referred elsewhere for reviews (Gowen et al. 2000; Rodan and Martin 2000).

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Macrophages in the Central and Peripheral Nervous System

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Abstract In the central and peripheral nervous system there are several distinct populations of macrophages. In the brain there are macrophages in the parenchyma and meninges, and there are macrophages associated with the vasculature. The macrophages in the brain parenchyma, the microglia are highly atypical with a distinct morphology and downregulated phenotype. The molecular mechanisms that underpin this unusual phenotype are being unravelled. Microglia rapidly respond to perturbations of their microenvironment and become activated in almost all brain pathologies. There is considerable interest in the possible role that activated microglia may have in brain and spinal cord pathology. The perivascular macrophages abutting the brain vasculature are also highly specialised macrophages and play an important role in communication between systemic inflammation and the brain. There is still much to learn about the role of macrophages in nervous system injury and repair.

Keywords Brain, CNS (central nervous system), Downregulation, Macrophage, Microglia, Neurodegeneration, Perivascular, PNS (peripheral nervous system), Spinal cord

In the central and peripheral nervous system there are large numbers of resident macrophages. These reside in different compartments of the nervous system, and the local microenvironment has a profound effect on their phenotype.

These macrophage populations have different roles in local homeostasis and respond differentially to injury and infection. It is now recognised that inflammation may contribute to the outcome of diverse neurological conditions including stroke, acute traumatic injury, HIV-1 associated dementia and Alzheimer's disease (Perry 1994). In all these conditions it is the macrophage that lies centre stage, and understanding how these cells contribute to brain damage and repair remains a significant challenge. Studies of the phenotype, the regulation of phenotype, and function of these different resident populations will aid in elucidating how these mononuclear phagocyte populations contribute to these diverse disease states.

1
Macrophages of the Central Nervous System

1.1
Microglia

The most abundant macrophage in the central nervous system (CNS) is the microglia. These cells have a distinct morphology (Fig. 1) and a distinct phenotype. The phenotype of the microglia in the normal adult CNS is best described as being in a downregulated or switched-off state (Perry and Gordon 1991). For example, microglia express low levels of major histocompatability complex (MHC) class I and II, low levels of CD45 (Streit et al. 1989) and low or undetectable levels of the scavenger receptor (Bell et al. 1994). The microglia do express F4/80, complement receptor type 3 (CR3) and Fc receptors (Perry et al. 1985). It is likely that the unusual morphology and the atypical phenotype gave rise to the debate as to whether microglia were or were not cells of the mononuclear phagocyte lineage (Ling and Wong 1993). However, numerous studies using bone marrow chimeras and specific panels of antibodies show that these cells are indeed the resident macrophages of the brain parenchyma (see Perry et al. 1993 for review). While there is little doubt that the microglia are of mononuclear phagocyte lineage, the precise origin and time of entry of mononuclear phagocytes into the CNS remains to be clarified (Kaur et al. 2001).

The microglia are present throughout the rostrocaudal axis of the adult CNS. They are more abundant in grey than white matter, and the density and morphology varies depending on their precise location (Lawson et al. 1990). Despite the marked neurochemical differences that exist from one brain region to the next, the phenotype of the parenchymal microglia, as judged by their morphology and expression of cell surface antigens, is rather uniform.

1.1.1
Phenotype

While numerous studies have documented the unusual phenotype of the microglia and the cell surface and cytoplasmic antigens that are, or are not, expressed

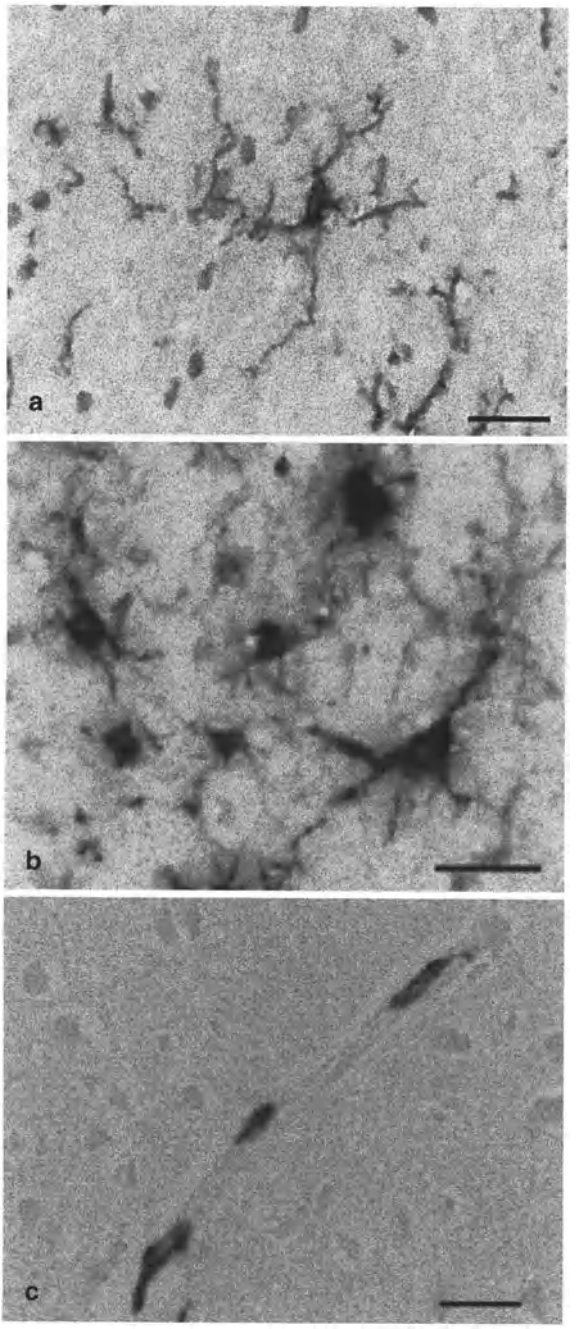

Fig. 1a–c Photomicrographs to illustrate complement type 3 expression on normal microglia (*upper*) and activated microglia (*middle*) in the rat brain. Perivascular macrophages are revealed by the monoclonal antibody ED2 (*lower*). *Scale bar*=20 μm

by these cells, the key issue is to discover the mechanisms that regulate their phenotype. What is it about the CNS microenvironment that so effectively downregulates the macrophage? One approach to this problem has been to investigate the factors that produce a microglia morphology or phenotype in vitro. Not surprisingly given the highly ramified nature of adult microglia (Fig. 1), the isolation of these cells from adult brain has not been routinely carried out, although it is possible (Ford et al. 1996). In the main, those studying microglia in in vitro studies have isolated immature microglia from neonatal brains, a time when the cells are less ramified and greater yields are obtained. However, the cells isolated from neonates, particularly when kept in culture for any length of time, have rather few similarities to microglia of the adult CNS. Indeed they have the phenotype of a typical macrophage, apart from some subtle morphological specializations (Giulian et al. 1995). To investigate the factors that might induce the microglia phenotype, various co-culture systems have been studied. Sievers and colleagues (Schmidtmayer et al. 1994; Sievers et al. 1994) have demonstrated that astrocytes will induce microglia morphology in macrophages derived from brain, spleen or blood and induce some aspects of the phenotype of the microglia. However, the astrocyte-derived cell surface, or secreted molecules, that may be responsible for the phenotype have not been isolated. A number of other systems have also been used to study factors that induce the microglia phenotype in vitro, but these have largely depended on morphological criteria and have not defined precisely the criteria for deciding that a macrophage in vitro actually represents the microglia phenotype in vivo.

At least part of the downregulated phenotype may come about as a consequence of immune regulatory molecules secreted by astrocytes or other cells in the CNS. Low levels of transforming growth factor $\beta 1$ (Kiefer et al. 1995) and interleukin-10 (Strle et al. 2001) are expressed in the normal CNS and they may contribute to microglia downregulation. There is evidence that neuronal activity is involved in the regulation of microglia phenotype, since blockade of neural activity in co-cultures leads to the upregulation of MHC class II expression by microglia (Neumann et al. 1996), an effect shown to be mediated by neurotrophins (Neumann et al. 1998). Recently, it has been shown that the interaction between CD200, expressed on neurons, and CD200R expressed by macrophages is involved in the regulation of microglia phenotype. Microglia in the CNS of mice lacking CD200 were found to have a more activated phenotype and formed focal clusters of microglia, although this was by no means ubiquitous (Hoek et al. 2000). The absence of CD200 also resulted in more rapid induction of experimental allergic encephalomyelitis. Other receptor–ligand interactions of this sort may be critically involved in the microglia phenotype (Barclay et al. 2002). It is known that the extracellular matrix of the CNS is highly atypical, being dominated by proteoglycans (Bandtlow and Zimmermann 2000), and that macrophages are differentially activated depending on the substratum to which they adhere. In an adhesion assay in which macrophages were allowed to adhere to brain sections a novel monoclonal antibody that blocked macrophage adhe-

sion to brain sections but not to spleen suggested the presence of a novel adhesive interaction (Brown et al. 1998).

In addition to the ligand–receptor interactions between microglia and components of the brain microenvironment, which may downregulate these cells, it may also be the absence of stimuli that play a part in the atypical phenotype. In regions of the CNS where the blood–brain barrier is absent and the macrophages are exposed to serum products, such as in the circumventricular organs, the resident brain macrophages are more activated (Perry et al. 1992).

1.1.2 Function

Numerous functions have been ascribed to the microglia, but definitive evidence is largely lacking. During development of the CNS, at least half of the neurons and glia generated in the embryo will not survive into adulthood, and at least some proportion of these cells undergoing apoptosis are phagocytosed by the microglia (Perry et al. 1985). Although it has been suggested that macrophages invading the developing brain may be in some way essential for CNS development, this seems unlikely. A comparison of the distribution of the apoptotic cells and the immature microglia reveals that these macrophages are opportunistic phagocytes rather than attracted to the apoptotic cells (Ashwell 1991). The PU.1-null mouse, which lacks myeloid cells, is viable until at least several weeks postnatally and major abnormalities of the CNS have not been reported (McKercher et al. 1996). This is not to say that there are no abnormalities of the CNS in these mice and results from more detailed studies will be of interest, particularly in the organisation of the fibre tracts where large numbers of macrophages invade the immature brain (Innocenti et al. 1983).

One structure in which the invading macrophages do appear to play an essential developmental role is in the vasculature of the eye (Lang and Bishop 1993). When macrophages were eliminated from the anterior chamber of the eye, the hyaloid artery and pupillary membrane were abnormally persistent (Diez-Rous and Lang 1997). These studies also showed that the macrophages do not just remove the cells of the vasculature but actively induce the endothelial cells to undergo apoptosis. Whether the macrophages are actively involved in remodelling of vasculature elsewhere or other components of the developing CNS remains to be established.

In the normal, healthy adult brain parenchyma, the role of microglia in brain homeostasis is unclear. Although there has been speculation that they may be involved in synaptic modelling, there is little evidence to support this except in the rather special circumstance of the neural lobe. In this structure, resident macrophages phagocytose the endings of hypothalamic magnocellular neurons where they abut the capillaries of the neural lobe (Pow et al. 1989). When the numbers of microglia processes in the neuropil are viewed in the context of the density of synapses, it is hard to envisage these cells playing a significant role in ongoing CNS synaptic plasticity.

The most important role for the microglia is that of first line of defence against injury and infection. It is now well documented that following any injury or pathology of the brain the microglia alter their levels of antigen expression and their morphology (Kreutzberg 1996). The more severe the disturbance, the more radical are the changes in the microglia morphology and phenotype but these cells are typically referred to as activated microglia (Fig. 1, middle). There is considerable interest as to how these cells, or other macrophage populations in the CNS, contribute to the outcome of diverse neurological diseases. In all conditions of acute or chronic neurodegeneration the microglia become activated, but not surprisingly there is no simple relationship between morphological activation and the associated cytokine profile (Walsh et al. 2000; Perry et al. 2002).

One key issue is the role of the microglia in antigen presentation and whether, for example, the upregulation of MHC class II leads to antigen presentation to CD4 T-lymphocytes and propagation of an immune assault on the CNS. There remains some controversy in this area but the weight of the evidence suggests that microglia are rather poor antigen-presenting cells (reviewed in Perry 1998), although other views have been expressed (Aloisi 2001). The normal brain parenchyma lacks dendritic cells and it appears that it is the perivascular macrophages that present antigen to T-lymphocytes patrolling the CNS (see below).

1.2 Perivascular Macrophages

A long overlooked population of CNS macrophages is that closely associated with the vasculature, the perivascular macrophages. These cells lie adjacent to the cerebral endothelial cells behind the blood–brain barrier but separated from the CNS parenchyma by a basement membrane (Graeber 1989) and are present throughout the rostrocaudal axis of the CNS. The perivascular macrophages have a simpler morphology than the microglia (Fig. 1, lower) and also a more activated phenotype. They express readily detectable levels of MHC class I and II CD45 (Streit et al. 1989) and in the normal mouse brain express the scavenger receptor (Mato et al. 1996) which is absent from the normal microglia. In the rat the monoclonal antibody, ED2 was found to be a selective marker of the perivascular macrophages (Graeber et al. 1989).

These cells are not only more activated than the microglia, they also turn over more rapidly. Data from a number of bone marrow chimera studies suggest that a significant proportion is replaced over a period of 3–6 months (Hickey et al. 1992). The important point in this regard is that monocytes are continually trafficking across the normal intact blood–brain barrier, which has implications for understanding how a number of intracellular pathogens, including HIV-1, may enter the CNS.

1.2.1 Function

One might expect from the location of these that cells they are well placed to respond to immune-activating molecules in the blood and are also the first cells that activated T lymphocytes will encounter on crossing the cerebral endothelium. Following a peripheral challenge with endotoxin [lipopolysaccharide (LPS)], to mimic a peripheral infection, it is the perivascular macrophages that first respond by increased synthesis of inhibitory factory $\kappa B\alpha$ and cyclooxygenase-2 (Cox-2) (Nguyen et al. 2002). This sensitivity of the perivascular macrophages relative to the microglia is also seen following intracerebral challenge with LPS or pro-inflammatory cytokines. The perivascular macrophages rapidly upregulate their synthesis of Cox-2 while the microglia do not (Minghetti et al. 1999). These cells play a major role in signalling between the periphery and the brain in the induction of fever and sickness behaviour that accompany systemic infection (Konsman et al. 2002).

The fact that perivascular macrophages constitutively express MHC class II molecules has led to the suggestion that these are the major APCs of the CNS. Studies by Hickey and Kimura (1988) show that these cells have the capacity to present antigen to encephalitogenic $CD4^+$ T lymphocytes. The selective isolation and study of perivascular and meningeal macrophages, as distinct from microglia, also shows that the $CD45^{high}$ population are competent APCs while the $CD45^{low}$ microglia are not (Ford et al. 1996). The observations raise the important question as to whether the perivascular macrophages are akin to, or are, dendritic cells of the CNS with the capacity to migrate from the brain compartment to lymphoid organs. At the present time there is little evidence to support the idea that there are dendritic cells in the perivascular space, or within the parenchyma of the brain. Two lines of evidence suggest that dendritic cells are present in the meninges and choroid plexus but not in the perivascular space or brain parenchyma.

In the rat the monoclonal antibody 0X62 recognises an integrin restricted to a population of dendritic cells and $\gamma\delta$ T lymphocytes (Brenan and Puklavec 1992). Using this antibody it has been shown that there are $OX62^+$/MHC class II^+ in the meninges and choroid plexus (Matyszak and Perry 1996; McMenamin 1999) but they are absent from the brain parenchyma. However, the presence or absence of any single antigen is not sufficient to define a cell as a dendritic cell, it is the functional capacity that is the key. Functional studies in vivo show that there are no dendritic cells in the brain parenchyma.

The microinjection of heat-killed mycobacterium bacillus Calmette-Guérin (BCG) into the ventricles, or on to the surface of the brain, gives rise to a typical overt delayed-type hypersensitivity (DTH) response. However, when the BCG is delivered in such a manner as to restrict it to the brain parenchyma it may reside there for many months undetected by the immune system (Matyszak and Perry 1995). Similar experiments have been performed using influenza virus (Stevenson et al. 1997). These simple experiments demonstrate that neither

perivascular macrophages nor microglia are able to phagocytose the BCG or virus and with these antigens then migrate to the lymphoid organs to initiate a primary immune response. In contrast, it has been shown that soluble antigen when delivered to the brain parenchyma, and with the appropriate precautions being taken to limit the delivery to the parenchyma, will rapidly drain via the perivascular spaces to the cervical lymph nodes (Cserr and Knopf 1992). The soluble antigen draining to the periphery results in an effective antibody response to the delivered antigen (Gordon et al. 1992). The functional significance of the drainage of soluble antigens, some of which are likely to be potentially immunogenic CNS antigens, is of interest in the maintenance of tolerance to CNS antigens.

1.3
Macrophages of the Meninges and Choroid Plexus

In the meninges, the membranes covering the brain, and within the stroma of the choroid plexus there are large numbers of macrophages. Although these macrophages lie outside the CNS itself, it is clear that these cells have the potential to play a significant part in immunological reactions in the CNS. These macrophages have a more activated phenotype than the microglia and perivascular cells and there are also some dendritic cells (Matyszak et al. 1992; Matyszak and Perry 1996; McMenamin 1999). These macrophage populations are involved in both innate and acquired immune reactions (see above) in the CNS.

2
Macrophages of the Peripheral Nervous System

Within the endoneurium of peripheral nerves there are resident macrophages which constitute about 5% of the total cell population. There are also large numbers of macrophages in the membranes covering the nerves and peripheral ganglia (Braun et al. 1993). It is not known whether these macrophage populations play any significant role in peripheral nervous system (PNS) homeostasis, but they are likely involved in the response to nerve injury. There has been considerable interest as to whether the monocytes that invade a peripheral nerve after nerve injury play a part in the regeneration response (Lazarov-Spiegler et al. 1998). However, data show that macrophages phagocytose the myelin and axon debris in the distal segment of the injured nerve but play a rather minor role in the regeneration of the peripheral nerve fibres; it is the peripheral glial cell, the Schwann cell, that is key to successful regeneration (Hughes and Perry 1999).

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Innate Recognition of Viruses by Macrophage and Related Receptors: Potential Ligands for Antiviral Agents

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Abstract Resident tissue macrophages are ideally placed for a role in the first line of defence against invading viruses and other microorganisms. Binding of viruses to macrophages can occur via a variety of molecules including the direct use of proteins, lipids and oligosaccharides as receptors, as well as opsonic recognition of viruses by the macrophage. Often multiple surface components are involved in virus–macrophage interactions, and interestingly a number of lectin receptors are expressed on macrophages for which viral ligands have been proposed. This review focuses on the initial viral attachment to macrophages and describes the interactions of certain macrophage lectin, proteoglycan and Toll-like receptors with viruses. The study of the role of pattern recognition receptors in the macrophage response to viruses may reveal new features of viral pathogenesis and some of the interactions could provide targets for antiviral agents.

Keywords Attachment, Collectin, Lectin, Macrophage, Proteoglycan, Receptor, Virus

Abbreviations

APC	Antigen-presenting cell
CMV	Cytomegalovirus
CRD	Carbohydrate recognition domain
DC	Dendritic cell
GAG	Glycosaminoglycan
HSV	Herpes simplex virus
LPS	Lipopolysaccharide
MBL	Mannan-binding lectin
MR	Mannose receptor
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
RSV	Respiratory syncytial virus
TLR	Toll-like receptor

1 Introduction

Innate immune mechanisms are critical in limiting the spread of infection within the host and provide protection while specific immunity develops. These processes are rapid and non-specific in the sense of being active against a broad spectrum of microbial pathogens. Innate immunity to pathogens is thought to be triggered by pattern recognition receptors (PRR) on antigen-presenting cells (APC) that detect and respond to conserved structural motifs on invading microorganisms. Toll-like receptors (TLR) have been proposed to play an essential role in linking innate and acquired immunity through their ability to fulfil the role of the PRR on the APC, as they respond to defined lipid, protein and nucleic acid components of microorganisms and signal cytokine production and costimulatory molecule induction (Akira et al. 2001). Other PRR which recognise pathogen-associated motifs include complement, CD14, calcium-dependent (C-type) lectins [mannose receptor (MR), mannan-binding lectin (MBL), DC-SIGN], and scavenger receptors. Much of our knowledge of the interaction and function of innate immune receptors in microbial infections has to date focussed on interactions with bacteria, mycobacteria, fungi and parasitic pathogens, whereas the role of these receptors in response to viruses is largely unexplored.

Macrophages are a major cellular element in the clearance and inactivation of viral pathogens. Resident tissue and blood macrophages are ideally placed to play an important role in mediating the innate interaction between the host and virus. Their ability to phagocytose free virus, or virus opsonised by serum collectins, complement or antibody, and hence clear virions from the circulation, constitutes an early line of defence for reducing virus load. Subsequent to phagocytosis, the associated intracellular killing of viruses contributes to non-productive viral clearance. In addition to immediate clearance of invading viruses,

macrophages phagocytose virus-infected cells, along with apoptotic cells or necrotic cells, earning their reputation as the most efficient phagocytes in the body.

Viral interaction with, and infection of, macrophages can stimulate these cells to release a number of proinflammatory and immunoregulatory cytokines and chemokines, along with proteolytic enzymes and oxygen radicals in vitro and in vivo (Julkunen et al. 2000; Guidotti and Chisari 2001). These factors act to limit virus spread and initiate a series of immune reactions including the attraction of lymphocytes, neutrophils and mast cells to the site of virus production, inviting them to play a role in host defence. The production of interferon-α and interferon-β by macrophages can further limit virus spread through its ability to induce an antiviral state in uninfected cells.

The activation of the innate immune response is a prerequisite for the triggering of acquired immunity. It is through modulating the threshold of activation of adaptive antigen-recognition receptors and by inducing key costimulatory molecules and cytokines, that innate immunity may shape the nature of the response and determine to which antigens the acquired immune system responds (Fearon and Locksley 1996). Recognition of antigenic arrays or pathogen-associated molecular patterns (PAMPs) by PRR (Medzhitov and Janeway 1997) on macrophages and on dendritic cells (DC) has been hypothesised to induce activation of defence mechanisms in the cell and the maturation of the cell into an efficient APC that subsequently attracts and activates the antigen-specific T cells essential for an adaptive immune response (Fearon and Locksley 1996; Janeway 1989). Mononuclear phagocytes, especially DC, act as APC, taking up, processing and delivering viral antigens to T cells in regional lymph nodes. While DCs are generally regarded as being the most efficient APC, macrophages can also present viral antigens to primed T lymphocytes. Through their function as APCs and their secretion of inflammatory and immunoregulatory cytokines, macrophages form an important bridge between innate and acquired antiviral immune responses.

In addition to their role in viral defense, macrophages are targets for viral infection and may provide an infectious reservoir for persistent viruses such as lentiviruses and cytomegaloviruses (CMV). For example, the cytopathic effects observed following HIV-1 infection of macrophages appear to be minimal, and infected macrophages remain viable; hence, macrophages may provide a mechanism of viral dissemination. In a model system of rhesus macaque monkeys infected with a simian immunodeficiency virus (SIV)/HIV-1 chimera, tissue macrophages were identified as the principal reservoir of virus after the depletion of $CD4^+$ T cells (Igarashi et al. 2001). This system, which is thought to be analogous to the late stages of HIV-1 infections in humans, showed that tissue macrophages sustained high plasma virus loads, implicating tissue macrophages as an important reservoir of virus in vivo. Measles and mumps viruses also avoid killing within macrophages, enabling these viruses to utilise the migratory properties of these cells to disseminate to the respiratory tract and salivary glands, respectively.

Table 1 Examples of some virus receptors found on macrophages

Receptor	Virus	Reference(s)
Protein receptors		
CD4	HIV	Collman et al. 1990; Weiss 2002
Poliovirus receptor	Polioviruses	Mendelsohn et al. 1989; Freistadt and Eberle 2000
Undefined macrophage membrane proteins	Dengue virus	Moreno et al. 2002
TLR3	ds viral RNA	Alexopoulou et al. 2001
Carbohydrate receptors		
Sialic acid-containing oligosaccharides	Influenza virus	Wilson et al. 1981; Weis et al. 1988
Heparan sulphate	Human CMV	Compton et al. 1993
	HSV	Herold et al. 1994
Antibody-dependent enhancement of viral entry		
Fc receptors (via bound immunoglobulin)	Dengue virus	Daughaday et al. 1981
	West Nile virus	Peiris et al. 1981; Cardosa et al. 1986
	HIV	Takeda et al. 1988
	CMV	Inada et al. 1985
	Influenza virus	Ochiai et al. 1988; Tamura et al. 1991

See text for definitions of abbreviations.

Viruses generally infect cells by endocytosis (rather than phagocytosis) or by fusion with the plasma membrane. Entry of a virus is often a multistep process, including initial attachment of the virus to the target cell surface, followed by fusion between the viral and cellular membranes and culminating in the internalisation of the viral genome into the cytosol of the target cells. The extracellular receptors used by viruses for attachment and entry are, presumably, receptors that serve other functions in the host. In some cases, host molecules are discovered as virus receptors prior to determination of their natural ligands and functions in normal host physiology. Given that host proteins are being used by viruses for attachment, choosing such receptors as antiviral targets will provide the challenge to designers of anti-viral drugs, to block viral binding without interfering with the normal functions of the host cell receptors.

Virus particles have multiple avenues for engaging cells and can interact with many molecules on the macrophage cell surface. The composition of a virus allows it to bind in a more complex manner to macrophages, than, for example, molecules such as lipopolysaccharide (LPS). Binding of viral lipid to CD1 or to annexins can occur, as well as protein–protein and lectin-carbohydrate interactions, be they on the cell surface or in endocytic vesicles. Hence, multiple receptors may come into play when examining recognition of viruses by macrophages and, whether multistep or involving a single molecule, the interactions can have a range of possible outcomes. This review will principally focus on the initial vi-

Table 2 Soluble and cellular C-type lectin receptorsfor viruses

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Receptor	Location	Viral ligand	Reference(s)
MR	Most macrophages, immature DC	HIV (gp120)	Larkin et al. 1989; Curtis et al. 1992
		Influenza virus	Reading et al. 2000
DC-SIGN	Immature DC, lung and placental macrophages	HIV-1, HIV-2	Curtis et al. 1992;
		SIV	Geijtenbeek et al. 2000; Pohlmann et al. 2001
		Ebola virus	Alvarez et al. 2002
DC-SIGNR	Endothelial cells in liver and lymph node	HIV-1, HIV-2	Pohlmann et al. 2001
SP-A	Lung surfactant	Influenza virus	Benne et al. 1995; Benne et al. 1997
		RSV	Ghildyal et al. 1999; Hickling et al. 1999; Barr et al. 2000
		HSV	van Iwaarden et al. 1991, 1992
		CMV	Weyer et al. 2000
SP-D	Lung surfactant	Influenza virus	Hartshorn et al. 1994; Reading et al. 1997; Hartshorn et al. 2000
		RSV	Hickling et al. 1999; LeVine et al. 1999
MBL	Serum	Influenza virus	Anders et al. 1990; Hartshorn et al. 1993; Malhotra et al. 1994
		HIV	Ezekowitz et al. 1996

See text for definitions of abbreviations.

ral attachment to macrophages. A selection of macrophage molecules that function as receptors for a number of viruses will be discussed, including receptors involved in both defence mechanisms against viruses and those commandeered by viruses for entry and infection. Identification of cellular receptors for viruses and inhibition of macrophage infection by viruses is of particular interest, since this is an important target for clinical therapy and could be used to prevent viral infection and progression of disease.

2 Interaction of Macrophage Receptors With Viruses

The interaction of macrophages with viruses can be mediated by a diverse set of receptors. Table 1 gives some examples of the types of viral receptors found on macrophages. In some cases the expression of protein receptors can explain the species, tissue and cell tropism of a virus, whereas carbohydrate receptors tend to be more broadly expressed on different cell types and hence do not always directly account for the tropism of a virus. An important mechanism of entry into macrophages for some viruses is via complexes with subneutralising

amounts of virus-specific antibody, binding to Fc receptors. Macrophages may also be able to detect entry and replication of virus, using the TLR3 receptor that has recently been shown to recognise double-stranded (ds)RNA, the molecular intermediate associated with many viral infections (Alexopoulou et al. 2001).

A number of viruses bind to multiple receptors during the infection process, a fact which may be important in clustering of different receptors to generate novel molecular assemblies with distinctive properties. For instance, interactions with one receptor may be required to bring about conformational change or endocytosis required for fusion, or for binding to a secondary receptor. Distinguishing between binding of viruses to "productive receptors" (Dimmock 1982) that lead to infection and non-specific binding to cell surface receptors is a key issue that is further complicated by the hypothesis that such non-specific binding to the cell may be important in concentrating virus particles on the cell surface for subsequent interactions with a specific low-affinity host cell receptor.

2.1 Macrophage Lectin Receptors

Lectin receptors can recognise carbohydrate on the surface of micro-organisms, and so selectively alert the innate immune system to the presence of potential pathogens. Such foreign carbohydrate motifs are found on a variety of micro-organisms, and hence lectin receptors may bind to a number of different microbial pathogens. For example, the MR binds to yeast, HIV, influenza virus and a number of bacteria. While bacterial sugars are encoded for and produced by bacteria, viral sugars are, by contrast, produced by the host, for the most part. This provides a greater challenge for recognition and specific targeting of viruses by the immune system, which has evolved to recognise foreign molecules.

The roles of macrophage lectins in host defence against bacteria and yeasts have been well reviewed elsewhere (Linehan et al. 2000) and this review will focus on their known and possible interaction with viruses. Table 2 summarises known interactions between viruses and soluble and cellular C-type lectins, some of which are described in more detail in the following text. The soluble collectins are included in this discussion, although they are not macrophage receptors, as they have similar carbohydrate specificity to that of the macrophage mannose receptor, and they opsonise microbes for their uptake by phagocytes (Holmskov et al. 1994; Hoppe and Reid 1994; Ezekowitz et al. 1996).

2.1.1 Mannose Receptor

The MR (CD206) is an integral membrane C-type lectin expressed on the surface of alveolar and other tissue macrophages. A multilectin receptor, the MR has specificity for two groups of sugar ligands; the 8 carbohydrate recognition

domains (CRDs) define the specificity for mannose, fucose and N-acetylglucosamine (Taylor et al. 1990, 1992), while the N-terminal cysteine-rich domain of the MR binds to sulphated terminal N-acetylgalactosamine residues (Fiete et al. 1998; Martinez et al. 1999; Leteux et al. 2000). Capable of binding to a wide range of ligands, the MR is a prototypical PRR (Stahl and Ezekowitz 1998). It is a characteristic marker of macrophages but in addition it can also be expressed on DC (Dong et al. 1999) and selected endothelial cells.

The MR has been implicated as a major endocytic receptor in the infectious entry of influenza virus. The ability of influenza virus to infect macrophages was shown to correlate both with the levels of MR expression on the macrophage and with the level of mannose-containing oligosaccharide present on the haemagglutinin molecule of the virus (Reading et al. 2000). Furthermore, purified glycoproteins from influenza virus inhibited the binding of mannosylated bovine serum albumin (BSA), a ligand of the MR, to peritoneal macrophages. Periodate treatment of the viral glycoproteins to oxidise their carbohydrate, prior to their inclusion in the assay, reduced their inhibitory capacity, consistent with a direct interaction of the influenza virus glycoproteins with the lectin domains of the MR.

The envelope glycoprotein of HIV, gp120, is a heavily glycosylated molecule implicated in binding to the MR (Daughaday et al. 1981; Curtis et al. 1992). Carbohydrate represents approximately half of the molecular weight of gp120 (Allan et al. 1985; Ratner et al. 1985), and when grown in human T cells, the oligosaccharide moieties of gp120 comprise approximately 50% oligomannosidic species, with fucosylated complex- and hybrid-type oligosaccharides also present (Geyer et al. 1988), making this viral glycoprotein a good potential ligand for the MR as well as other lectin receptors. Evidence for the interaction of gp120 with the MR comes from binding studies which showed that gp120 binding to a MR immobilised on beads was inhibitable by mannosylated-BSA but not by soluble CD4 glycoprotein. Additionally, binding of mannosylated-BSA to human peripheral blood monocyte-derived macrophages was inhibited by recombinant gp120, but not by soluble CD4 (Larkin et al. 1989).

Given the wide range of bacteria, yeasts, parasites and mycobacteria recognised by the MR (Fraser et al. 1998, and references therein), it is surprising that so little work has investigated its interaction with viruses. Further research needs to be performed on examining viral ligands for the MR. It should also be noted that the use of mannosylated-BSA as a blocking agent in the work described above does not conclusively demonstrate that these viruses interact with the MR, due to the existence of other mannose-specific lectins on macrophages (Imamura et al. 1984; Fernandes et al. 1999). Available anti-human MR monoclonal antibodies and the recent development of monoclonal antibodies specific for the murine MR (L. Martinez-Pomares, personal communication) will be useful in clarifying viral interactions with this receptor.

2.1.2
DC-SIGN and Also DC-SIGNR

The type II membrane protein DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin; CD209) has one mannose-binding C-type lectin extracellular domain. This protein is proposed to function normally in binding to ICAM-2 expressed on endothelial cells, to promote transmigration of DC from the blood to lymphoid tissues, and in binding to ICAM-3 on resting T cells, contributing to T-cell activation (Geijtenbeek et al. 2000a,b). DC-SIGN is better studied on immature DC, where it is highly expressed (Geijtenbeek et al. 2000b) but is also found on macrophages (Soilleux et al. 2002). The related molecule, DC-SIGNR, is found on liver sinusoidal endothelium and endothelium of lymph node sinuses and placental villi, and its expression on macrophages has not yet been documented. Both of these lectins, DC-SIGN and DC-SIGNR bind to HIV-1, HIV-2, SIV and Ebola virus (see references in Table 2).

DC-bearing DC-SIGN have been proposed to play a role in the establishment of HIV infection and may be critical in acquiring virus and transmitting it to T lymphocytes. DC-SIGN does not act as a receptor for entry of HIV into cells, but facilitates viral infection in *trans* of target $CD4^+$ T cells (Geijtenbeek et al. 2000b; Pohlmann et al. 2001). While mannan very efficiently blocks transmission of HIV-1 by cells transfected with DC-SIGN, mannan (or DC-SIGN-specific monoclonal antibodies) only partially block HIV transmission by DC, suggesting that other factors may be utilised by the virus, in addition to DC-SIGN, for DC-mediated HIV-1 transmission to target cells (Wu et al. 2002b). Additional support for this hypothesis comes from the observation that DC from rhesus macaques do not express DC-SIGN and are still able to efficiently transmit primate lentiviruses (Wu et al. 2002a).

DC-SIGN-mediated transmission of HIV to T cells does not appear to require the ICAM-3 binding activity of the DC-SIGN molecule, and binding to HIV may not be mediated by a lectin interaction. Binding to ICAM-3 requires calcium and is dependent on glycosylation of ICAM-3, whereas in contrast, binding of DC-SIGN to the HIV-1 envelope glycoprotein is independent of N- and O-linked glycosylation (Geijtenbeek et al. 2002). A mutant of DC-SIGN that no longer bound to ICAM-3, but retained a specificity for gp120, was still able to mediate HIV-1 infection of T cells efficiently in *trans*, suggesting that virus transmission was not dependent on DC-SIGN interactions with ICAM-3. Thus, it appears that there are overlapping but distinct sites for DC-SIGN binding to ICAM-3 and to gp120. This may aid the development of inhibitors of HIV attachment to DC-SIGN that have reduced negative effects on the normal function and roles of DC-SIGN.

While research has focussed on DC-mediated transfer of HIV to T cells, now that macrophages have also been shown to bear DC-SIGN, it will be important to assess the degree to which they have roles in carrying virus from the periphery into lymph nodes.

2.1.3 Collectins

Endogenous lectins, including soluble collagenous C-type lectin members of the collectin family, have an important role in innate defence against respiratory viruses (Sastry and Ezekowitz 1993; Malhotra and Sim 1995; Crouch et al. 2000). These proteins are known to bind to influenza virus, respiratory syncytial virus (RSV), herpes simplex virus (HSV), HIV and CMV (see references in Table 1) and display antiviral activity in vitro and in vivo. Members of this family found in humans include serum MBL, and the lung surfactant proteins (SP)-A and -D. Collectins use multivalent lectin-like domains coupled to collagenous stalks for the recognition and opsonisation of microbes for uptake by macrophages. Much of the investigation of the interaction of collectins with viruses has been performed with influenza virus.

The in vitro antiviral activities of collectins against influenza virus include virus neutralisation and aggregation, opsonisation for contact with neutrophils, and lysis of virus-infected cells in the presence of complement (Reading et al. 1995; Hartshorn et al. 1997; Anders et al. 2001) and are consistent with a role for these molecules in first line host defence. MBL and SP-D bind to glycans on the influenza virus haemagglutinin (HA) and neuraminidase glycoproteins, whereas the interaction of SP-A with viruses involves binding of the viruses to SP-A-associated carbohydrates (Benne et al. 1995; van Iwaarden et al. 1991, 1992). Bovine SP-D has been shown to inhibit infectivity of rotaviruses through Ca^{2+}-dependent, mannose-inhibitable attachment to the major viral envelope glycoprotein (Reading et al. 1998b).

Only more recently have roles for collectins in innate defense against influenza virus been demonstrated in vivo. Studies on the sensitivity of a range of strains of influenza virus to collectin-mediated neutralisation revealed a marked inverse correlation between collectin sensitivity and the ability of a virus to replicate in the mouse lung after intranasal inoculation (Reading et al. 1997). Co-administration of mannose-containing oligosaccharides along with virus resulted in markedly increased replication of influenza A virus in the lung. Although MBL levels were undetectable in lavage from normal or influenza-infected mice, SP-D levels were detected and increased several fold after influenza A virus infection. Strains of influenza virus bearing higher levels of glycosylation on the HA molecule grew very poorly in the mouse lung, whereas the A/PR/8/34 strain, which grows to high titres in mouse lung, carries no glycans on the head of its HA molecule and is essentially resistant to neutralisation by SP-D and MBL. Overall, these results provided strong evidence that lung collectins, in particular SP-D, contribute significantly to containment of influenza infection in vivo.

There are many strong indications for roles of SP-D and SP-A in innate immunity. Reading et al. showed that compromise of SP-D, due to elevated glucose levels in the lungs of diabetic mice appeared to be the major factor contributing to the increased susceptibility of diabetic mice to influenza virus (Reading et al.

1998a). The study of infection in the SP-D knockout mouse is hampered by a phenotype sharing features of alveolar lipoproteinosis disease, with activated macrophages and accumulation of surfactant in the alveolar spaces (Botas et al. 1998; Korfhagen et al. 1998). Nevertheless, SP-D-deficient mice showed decreased clearance (compared to wild-type mice), of a heavily influenza virus (LeVine et al. 2001). In contrast to the proteinosis observed in the lung of the SP-D knockout mouse, the SP-A knockout mouse has only marginal defects in surfactant homeostasis and respiratory function, and studies on the immune response to RSV, adenovirus and other pathogens have confirmed its role as an innate immune protein (LeVine et al. 1999; Harrod et al. 1999; Lawson and Reid 2000). Influenza virus and RSV clearance from the lungs of SP-A knockout mice was significantly decreased compared to wild-type SP-$A^{+/+}$ mice and was associated with increased neutrophil and lymphocyte numbers in the bronchial alveolar lavage along with increased titres of proinflammatory cytokines in lung homogenates (LeVine et al. 1999, 2002).

Together these observations strongly indicate that the ability of these respiratory viruses to replicate in the lungs of mice is limited by lectin-mediated defence mechanisms. Furthermore, these studies identify viral carbohydrate as a ligand for recognition of virus by collectins of the innate immune system and are consistent with the hypothesis that glycosylation can affect virulence through mediating susceptibility to such innate mechanisms as collectins and phagocytic lectin receptors. Collectins can be thought of as soluble receptors that bind to PAMPs, to opsonise particles for uptake by macrophages; however, the receptors for collectins are still poorly defined. The antiviral activity of collectins against a number of enveloped viruses supports the hypothesis that collectins represent a general innate defence mechanism of natural resistance.

3 Other Lectin Receptors

Other lectin receptors have been described on the macrophage, and a number of novel lectin receptors have recently been discovered (Balch et al. 1998; Bakker et al. 1999; Bates et al. 1999; Matsumoto et al. 1999; Brown and Gordon 2001). For many of these molecules neither the sugar specificity nor a function has yet been determined. However, by extrapolation from observations with the MR, DC-SIGN and collectins, these lectin receptors are potential PRR that may bind a wide selection of micro-organisms, including viruses. Viral ligands for these macrophage receptors have not been identified as yet. Some receptors that were described initially on DC have subsequently been shown to have broader cell expression. For example, DC-SIGN was originally isolated from human placental cDNA library (Curtis et al. 1992) and was described as being exclusively expressed on DC (Geijtenbeek et al. 2000c); however, it is now realised that this receptor is also expressed on alveolar and placental macrophages, as well as $BDCA2^{+}$ plasmacytoid peripheral blood DC precursors in situ and in vivo (Soilleux et al. 2002).

Of note, DEC-205 (CD205) is topologically very similar to the MR and expressed on DC and macrophages. This multilectin receptor has 10 CRDs and appears to mediate uptake of glycosylated antigens, although to date no ligand specific for any of the CRDs in DEC-205 has been identified. The similarity of DEC-205 to the MR suggested that it too may be involved in recognition of pathogens and play a role in innate immunity and in antigen processing. Interestingly, none of the CRDs of DEC-205 have conserved the key amino acids involved in carbohydrate and calcium binding, consequently it is unlikely that the CRDs of this receptor have lectin activity (Inaba et al. 1995; Jiang et al. 1995; Swiggard et al. 1995).

Finally, viral glycoproteins themselves can also function as lectins, and mediate binding to cell-surface oligosaccharides. Influenza virus initiates infection by binding of the viral HA glycoprotein to terminal N-acetylneuraminic (sialic) acid-containing receptors on the cell surface (Wilson et al. 1981; Weis et al. 1988). The receptor specificity of the influenza virus HA has been well studied and depends on a number of amino acid residues in the receptor-binding pocket of the HA which are involved in either direct or stabilising interactions with sialic acid. Experiments have also suggested that the HIV gp120 glycoprotein may have lectin-like properties. Based on studies examining transcellular transport of gp120-coated particles, Kage et al. suggested that the gp120 glycoprotein of HIV-1 may contain a lectin-like domain that interacts with mannosyl residues on the mucosal surface (Kage et al. 1998).

3.1 TLR

Janeway proposed that leukocytes must have molecules that are able to recognise antigenic arrays or patterns that are generic to micro-organisms and absent from host cells (Janeway 1992). Such PAMPs include bacterial LPS, peptidoglycan and lipoteichoic acid of gram-positive bacteria, unmethylated CpG deoxynucleotide motifs in prokaryotic DNA, and mannans on fungi and viruses, and are recognised by both soluble and cell-associated PRR. Members of the TLR family function as PRR in mammals and have been intensively investigated recently. TLR discriminate between a variety of microbial products, for example TLR2 confers responsiveness to lipoproteins and several gram-positive bacteria, TLR4 binds to LPS, TLR5 mediates responsiveness to flagellin and TLR9 recognises unmethylated DNA containing CpG motifs (CpG-DNA) (Akira et al. 2001).

To date, only TLR4 and TLR3 have been implicated as being involved in innate immunity to viruses. The proinflammatory cytokine response to the fusion protein of RSV in vitro, and cellular responses and viral clearance following intranasal infection with live virus were reduced in mice mutated in the gene encoding TLR4 (Kurt et al. 2000; Haynes et al. 2001). In addition, TLR4 has recently been identified as one of the components mediating activation of B cells in response to the retroviruses mouse mammary tumour virus and Moloney murine leukaemia virus (Reading et al. 1998a). The recognition of dsRNA by TLR3

induces activation of nuclear factor (NF)-κB (in T cells and macrophages) and the production of type 1 interferon (Alexopoulou et al. 2001), suggesting a role for this TLR in the antiviral response; however, this remains to be established. It is of interest that influenza or Sendai infection of human macrophages, or exogenously added interferon-α, enhanced mRNA expression of TLR1, TLR2, TLR3 and TLR7, and downregulated TLR5 mRNA (Miettinen et al. 2001), suggesting that control of TLR expression by type 1 interferon may be a novel mechanism by which interferon can modulate the innate immune response.

Subversion of a host defence mechanism by viruses gives another indication of the importance of that defence mechanism. Vaccinia virus carries two proteins products, A46R and A52R, with similar amino acid structure to Toll/interleukin (IL)-1 receptor domains (that define the TLR family), through which IL-1 and TLR4 signalling can be inhibited (Bowie et al. 2000). The evolution of these antagonistic proteins by vaccinia highlights the importance of TLR-mediated mechanisms in immune defense against this virus.

TLR recognise a heterogeneous variety of ligands; however, it is not known what viral structure might be recognised by TLR. It may be that other molecules and/or receptors assist TLR recognition of viruses, analogous to the manner that cellular CD14 or secreted MD-2 bind to LPS to facilitate its interaction with TLR4. A functional receptor complex may be involved in TLR interaction with virus, which may contain lectins, chemokines, etc. that coordinate to facilitate recognition, binding and signal transduction in response to virus binding. Some TLR have been shown to be located intracellularly, in which case co-receptors may be essential for delivery of virus, or viral antigens, to the TLR. Alternatively the natural pathway of virus infection may result in the virus meeting an intracellular TLR. Future studies in this area should clarify viral interactions with TLR.

3.2 Proteoglycans

Proteoglycan molecules, present on the surface of mammalian cells, and in the extracellular matrix, are composed of a membrane-linked protein core with attached, variously charged sulphated glycosaminoglycans (GAGs). Proteoglycans exist as both pure molecules, which have either only heparan sulphate or chondroitin sulphate GAG chains, and those with mixtures of different GAGs. The GAG chains consist of alternating residues of an amino sugar and an uronic acid, and as the chains polymerise, varying amounts of sulphation and epimerisation result in a large degree of heterogeneity amongst proteoglycans. Proteoglycans can be separated based on the charge characteristics of the GAG chains, with greater levels of sulphation corresponding to a higher charge. Heparin is a highly sulphated version of heparan sulphate and one of the most highly sulphated GAGs.

Proteoglycans on the cell surface are used by a number of viruses as cellular adhesion receptors, with at least 10 human pathogenic virus infections implicat-

ed in binding to heparan sulphate (Rostand and Esko 1997; Wadstrom and Ljungh 1999; Bose and Banerjee 2002). For some viruses the initial binding to heparan sulphate-containing proteoglycans appears to function primarily to concentrate the virus particles at the cell surface, before interaction with additional, higher affinity receptors. This is the case for foot-and-mouth disease, which has a primary interaction with heparan sulphate followed by binding to the RGD motif of the $\alpha_v\beta 3$ integrin (Jackson et al. 1996). Other viruses, such as HSV, appear to be able to utilise heparan sulphate directly as an entry receptor (Shukla et al. 1999).

HIV and CMV are examples of viruses for which primary binding to cell-surface GAGs appears to assist viral interaction with secondary receptors required for entry. Heparan sulphate on the cell surface is also able to initiate binding of CMV (Neyts et al. 1992; Compton et al. 1993). A soluble form of the human CMV glycoprotein B (gB) showed 40% reduced binding to CHO cells lacking heparan sulphate proteoglycans and to fibroblast cells treated to remove heparan sulphate. However, an undefined non-heparin component of binding remained, suggesting the presence of another class of human CMV cellular receptors (Boyle and Compton 1998). Heparan sulphate has also been proposed to be involved in the initial attachment of HIV-1 to target cells, prior to the known required interaction between gp120 and CD4 and an appropriate chemokine receptor. Only HIV-1 isolates containing highly positively charged V3-loop sequences showed reduced infectivity in cells lacking GAGs (Zhang et al. 2002). This marked strain-dependent difference in the requirement for target cells to express cell-surface heparan sulphate (Ohshiro et al. 1996; Mondor et al. 1998) supports the hypothesis that attachment is likely to be mediated by electrostatic interactions, predominantly between the charged V3 domains of gp120 and cell-surface heparin (Roderiquez et al. 1995).

Evidence to highlight the importance of a primary interaction between HIV and GAGs for the infectious process has recently been highlighted. The gp120–CD4 interaction alone is not thought to be sufficient for tight attachment of HIV to cells such as macrophages, microglia and DC (Sonza et al. 1995; Dick et al. 1997), and the additional binding of virus to cell-surface GAGs, along with other adhesion receptors, is thought to be required for efficient infection of these cells (Ugolini et al. 1999). This was clearly shown when HIV-1 no longer attached to monocyte-derived macrophages after heparitinase treatment, which removes all the cell surface heparan sulphate chains but leaves CD4 unaltered (Saphire et al. 2001).

In the case of HSV-1 and HSV-2, the interaction of glycoproteins gB and gC with cell surface heparan sulphate has been more thoroughly studied. Both clinical and laboratory isolates of alphaviruses initially attach to cells via interactions of gB and gC viral glycoproteins with heparan sulphate proteoglycans (Herold et al. 1991, 1994; Lee and Fuller 1993), and in particular, the sulphate groups at C-2 of the uronic acids and the carboxyl groups were critical for gB binding to heparin (Herold et al. 1995). In the absence of cell-surface heparan sulphate, virus entry is very inefficient. However, with time virus adherence to

cells can become irreversible by heparin, suggesting that additional interactions with non-heparan sulphate receptors can occur. Further to their role in viral binding, involvement of proteoglycans in viral entry has been suggested. Binding of gB to heparan sulphate led to fusion of the viral envelope with the host-cell plasma membrane and syncytium formation (Shieh and Spear 1994). Subsequent to virus attachment to heparan sulphate, the viral gD glycoprotein, in concert with gB, gH and gL, interacts with any one of several co-receptors (Spear et al. 2000), to facilitate entry of HSV by a fusion process. Interestingly, one of the classes of co-receptors that gD can interact with to initiate HSV-1 entry are sites in heparan sulphate generated by the action of specific 3-O-sulphotransferases (Shukla et al. 1999).

Both the choice of specific macrophage subsets, and macrophage activation status, can significantly alter expression levels of many receptors on macrophages. For example, freshly isolated monocytes express very low levels of heparan sulphate GAGs, whereas macrophages derived by culture on plastic for 10 days express high heparan sulphate GAG levels (Clasper et al. 1999; Saphire et al. 2001). This may explain why the susceptibility to infection with HIV of freshly isolated monocytes increases during maturation (Gendelman et al. 1986; Rich et al. 1992). Having varied and changing levels of virus receptors on macrophages both increases the complexities of macrophage-virus interactions, and may help explain the tissue-specific susceptibility of cells to viral infection, particularly when infection is mediated via receptors thought to be ubiquitously expressed. For instance, different tissues have been reported to exhibit different heparan sulphate monosaccharide sequences (Lindahl et al. 1998; Jenniskens et al. 2000). The recent data suggesting that specific heparan sulphate sequences are recognised by viruses give us the best clues yet to understanding the tissue and cell-specific tropism of viruses that utilise GAGs for cell attachment and entry (Liu and Thorp 2002). Further analysis of saccharide sequences and structure of GAGs, and viral interactions with them, should improve the understanding of their role in assisting viral infections and will facilitate the future development of intervention strategies.

4 Conclusions

The success of viral invasion is an important factor in determining disease severity, and tissue macrophages are in a position to pose barriers to the establishment and dissemination of virus infection. Viruses bind, both specifically and non-specifically, to a number of cell-surface receptors on macrophages; understanding the biology of host-cell receptors used by viruses for binding and entry into macrophages may reveal new features of viral pathogenesis and lead to new modalities for preventing disease caused by viruses. This review has focussed on macrophage receptors, including lectins and proteoglycans, which are utilised by a number of viruses (along with other intracellular parasites) for attachment and entry. Even though the attachment process is usually very specific,

the range of viruses bound by these receptors suggests a degree of pattern recognition, and the spectrum of viruses bound by these receptors is likely to broaden further still with continued research. The outcome of the interaction between viruses and macrophages depends on the state of macrophage differentiation, as it can affect the receptors expressed by the cell, with some viruses potentially utilising alternative receptors on individual or different cells. In particular this highlights the importance of extending studies performed using macrophage cell lines, to include the interaction of viruses with tissue macrophages (Turville et al. 2001), and also to study how receptor expression varies on the different populations of tissue macrophages. As we improve our understanding of the interaction of viruses with their cognate receptors, we improve the opportunity to identify new targets for rationally designed drugs, that are unique and selective for viruses and that minimise the risks of inopportune adverse effects on the host. Knowledge gained from research into the role of host cell molecules as virus receptors may also assist the future development of viral vaccines.

One approach used when designing antiviral agents is to target specific viral replication processes, as has been done in the development of reverse transcriptase and integrase inhibitors for antiretroviral therapy. An alternative approach is competition for the ligand recognised by the virus. This can either be relatively non-specific, as in the case of carbohydrate ligands, or greater selectivity can be achieved using specific protein reagents, such as antibodies. Polyanionic compounds, such as dextran sulphate, have been tested as anti-HIV therapeutic agents to block the non-specific interaction that has been proposed to play a role in attachment of the HIV-1 virion to the cell surface (Abrams et al. 1989; Stafford et al. 1997). Although relatively cheap, sulphated polysaccharides are not specific inhibitors of HIV viral binding, being able to inhibit other viruses also (Leydet et al. 1998). The rational design of sialic acid analogues has led to the development of Relenza and Tamiflu, which bind with high affinity to the influenza virus neuraminidase to inhibit release of virus particles from infected cells. In general, however, sugars and polyanionic compounds do not have high affinities for their ligands and their bioproperties are considered poor. Proteins tend to have higher affinities for their ligands and bind more specifically. In either case, one must hope that the binding site for the viral ligand differs from that of the physiological ligand of a receptor. The mapping of both virus binding and functional domains of receptors may allow targeting of specific areas of receptors for focussed design of antiviral agents; however, we will most likely have to hope for some functional redundancy if we are to successfully target host receptors to prevent viral infection.

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Macrophage Immunity and *Mycobacterium tuberculosis*

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Abstract Tuberculosis (TB) is rivalled only by the acquired immunodeficiency syndrome (AIDS) as a communicable cause of death. Yet of an estimated 2 billion individuals who have been infected with the pathogen *Mycobacterium tuberculosis* (*Mtb*), less than 10% will develop disease. For the remainder, natural

immunity appears sufficient to limit bacterial growth. An integral component of host protection to TB is the activated macrophage. *Mtb* recognition, phagocytosis, vacuolar trafficking and redox-based killing are all enlisted as part of this cell's anti-tubercular arsenal. When assembled together with lymphocytes and stromal elements as part of the tuberculoid granuloma, macrophages also provide a physical constraint to further dissemination. The liaison between macrophages and T cells in particular forms much of the current basis of vaccination in immunologically naive subjects. Recent experimentation with post-exposure vaccines, however, suggests that cellular immunity may not be fully elicited by the existing single-dose regimen. New approaches that embrace small molecule chemistry to enhance or mimic macrophage effector mechanisms, or which sensitise *Mtb* to further immunologic insult, could help address this issue. Harnessing the macrophage as a therapeutic target could thus prove a useful adjunct to TB vaccination and chemotherapy in the future.

Keywords Cytokine, Macrophage activation, Microbial recognition, Mycobacterium tuberculosis, Tuberculosis, Vaccination

Abbreviations

AFB	Acid-fast bacilli
AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
CGD	Chronic granulomatous disease
CR	Complement receptor
FcR	Fc receptor
GPI	Glycosyl phosphatidylinositol
GTPase	Guanosine 5′-triphosphatase
IFN-γ	Interferon gamma
IL	Interleukin
RF	Interferon regulatory factor
Jak	Janus kinase
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LTBI	Latent TB infection
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
MR	Mannose receptor
Mtb	Mycobacterium tuberculosis
MGC	Multinucleated giant cell
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	Nuclear factor (NF)-κB
NRAMP	Natural resistance-associated macrophage protein
NO	Nitric oxide

NOS2	Inducible nitric oxide synthase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PG	Phagosome
PI_3P	Phosphatidyl inositol 3-phosphate
PL	Phagolysosome
PRR	Pattern-recognition receptor
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SNO	S-nitrosothiol
SP	Surfactant protein
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TNF	Tumour necrosis factor alpha

1 Introduction

1.1 The Relationship Between *Mtb* and Its Human Host: Ancient Origins, Modern Concerns

Exhumation of the 3,203 year-old tomb of Nebwenenef, high priest of Egyptian pharaoh Ramses II, uncovered the mummified remains of a young boy harbouring acid fast-bacilli (AFB) together with blood in his trachea (haemoptysis)—hallmarks of pulmonary tuberculosis (TB) (Zimmermann 1979). A further 31 mummies dated between 3700–1000 B.C. displayed angular kyphosis typical of Pott's disease, wherein TB causes long bone and spinal deformities (Morse et al. 1964). More recently, DNA specific for the pathogenic tubercle bacilli has been detected in the tracheobronchial lymph node of a 1,300-year old Peruvian corpse (Salo et al. 1994). So begins the paleopathological record of mankind's ongoing struggle with one of nature's most durable and successful pathogens, *Mycobacterium tuberculosis* (*Mtb*). This relationship, steeped in antiquity and human suffering, still accounts for nearly 2 million deaths and 8 million active new cases per year (WHO 2000). The introduction of effective chemotherapy in 1952 offered respite but patient non-compliance, governmental neglect and the advent of the AIDS epidemic have again helped raise the spectre of TB in the form of multi-drug resistant (MDR) strains. Add to this a global reservoir of clinically latent TB in nearly 2 billion infected people, an estimate which represents one-third of the earth's population (Dye et al. 1999), and the magnitude of the problem becomes palpable. Little wonder the search for novel anti-microbial drugs and protective vaccines has taken on renewed urgency (McKinney 2000). At the vanguard of this effort will be the quest for understanding how *Mtb* adapts to its human host, an interaction which focuses attention on the bacteri-

um's favoured dwelling and, paradoxically, its chief antagonist: the macrophage.[1]

1.2 Adversarial Profiles

Macrophages and *Mtb* share not only the legacy of co-evolution but also that of contemporaneous discovery. In 1882, the German physician Robert Koch delivered his landmark address to the Berlin Physiological Society in which he provided clear evidence for the tubercle bacillus being the aetiologic agent responsible for "consumption" (Koch 1882). In the same year, Russian-born zoologist Ilya (Elie) Metchnikoff had been watching through his microscope at home in Messina the wandering amoeboid cells in transparent starfish larvae, mobile cells which he knew could ingest solid particles from earlier experiments conducted in coelenterates (Metchnikoff 1880). Out of these observations grew the realisation that such cells may in fact help defend the organism against "noxious intruders" (Metchnikoff 1921). By placing a thorn under the larva's skin, Metchnikoff witnessed the amoeboid cells accumulate at the site of injury. This experience served as the basis for his seminal theory of phagocytosis, a postulate soon formally demonstrated by the ability of gut mesoderm in the water flea, *Daphnia*, to engulf the fungal ascospores of *Monospora bicuspidata* (Metchnikoff 1884). In turning his attention thereafter towards vertebrate immunity, Metchnikoff established the chief importance of macrophages in providing innate defence against invading micro-organisms (Metchnikoff 1905). Among his most significant and enduring findings was one of giant cells from resistant tubercular animals being capable of ingesting and killing Koch's causative agent (Metchnikoff 1888).

Today we appreciate that macrophages represent one of the most highly specialised lineages in all of metazoan immunity. Besides their marked phagocytic profile (shared to a lesser extent by certain other cell types, e.g. retinal epithelia), a number of additional characteristics have helped define their métier as host protectant and are especially relevant to TB. These include: (1) an abundant fixed tissue distribution, with sessile populations in nearly all organ systems, notably within alveoli (alveolar macrophages; AMs) as well as interstitial and intravascular macrophages originally sequestered as monocytes from the pulmonary microcirculation; (2) rapid serosal motility in response to chemotactic gradients generated within cavities such as the pleura; (3) multiple pathways for microbial killing and antigen presentation; and (4) an ability to co-opt other immunocytes to the site of infection, a task accomplished via the elaboration of some ten or more classes of secretory products (e.g. cytokines/chemokines, growth factors, coagulation factors, matrix proteins, bioactive oligopeptides, lipids, sterols, purines, pyridines and oxygen/nitrogen intermediates) (Nathan

[1] The term, "macrophage" as used here refers to all cells of the mononuclear phagocyte system, including monocytes.

1987; Gordon 1999). This degree of plasticity implies much in the way of co-ordinate regulation of a large number of genes. Indeed, macrophages are endowed with enormous biosynthetic capacity. Upon encountering *Mtb*, for example, murine macrophages regulate as many as 600–700 genes (of ~11,000 microarrayed) within the first 24 h (Erht et al. 2001). Some 10% of mRNA transcripts analysed in human macrophages are significantly induced (10- to 100-fold) as early as 6 h and up to 400-fold by 12 h post-infection (Ragno et al. 2001). Given that the vast majority of housekeeping genes remain unchanged and represent nearly 60% of the genomic total (Erht et al. 2001), a 20%–25% response of the remainder illustrates just what a potent signal *Mtb* is for the macrophage transcriptome. When accompanied by activating cytokines such as interferon gamma (IFN-γ), this genetic commitment may reach as high as 40% (Erht et al. 2001).

Why is *Mtb* such a powerful natural stimulus for macrophage activation? The answer, in part, lies with the unique physiology of the organism itself. *Mtb* is a non-motile, rod-shaped actinomycete closely related to saprophytic bacteria such as *M. smegmatis*. Despite staining poorly by the usual gram-stain procedure (owing to the impermeability of its thick, waxy cell wall), the tubercle bacillus is nonetheless grouped together with gram-positive bacteria, since they all possess a single, cytoplasmic membrane (McKinney et al. 1998). Like other gram positives, *Mtb* also shares a peptidoglycan wall yet augments it with an array of complex lipidoglycans, the latter of which may reflect the need to have resisted desiccation in an ancestral soil environment (Russell 2001). This lipid-rich structure avidly retains Carbol fuchsin dye even in the presence of acidic alcohol (hence the term, "acid fast") as well as providing the decidedly foreign (non-self) determinants against which cells of the immune system have evolved.

Where *Mtb* differs markedly from other gram-positive bacteria is in the slow rate at which it replicates: ~20–24 h in synthetic medium or infected mammals (McKinney et al. 1998). This indolent growth contributes to the chronic nature of the disease and undoubtedly provides a continuous source of immune activation, some of which is potentially injurious. Moreover, it imposes lengthy (6–9 month) treatment regimens and substantial obstacles for experimentation. It may also enable the bacilli to reside within the same human host for possibly decades before reactivating later in life (Lillebaek et al. 2002). Such tissue-adapted dormancy could involve metabolic shutdown as a result of the cell-mediated immune response which contains but does not immediately eradicate the infection. Whether *Mtb* acquires significant heritable changes as part of this adaptation remains unclear. Spoligotype and microarray analyses so far depict limited polymorphic diversity among clinical isolates, a genetic invariance which could have several explanations (Kato-Maeda et al. 2001). Multiple mutations and/or deletions, for example, may be poorly tolerated, or their low level representative of recent evolutionary dissemination among human hosts (Musser et al. 2000; Brosch et al. 2002). Alternatively, extant strain similarities may truly reflect a lengthy intracellular quiescence (Musser et al. 2000; Brosch et al. 2002), one which could account for the persistence of certain isogenic strains within communities of low endemicity or reinfection rates (Kato-Maeda et al. 2001).

Reactivation of latent TB occurs in ~5% of infected individuals, while in another 5%, "primary progressive" TB usually ensues within a year or two of transmission (McKinney et al. 1998; Parrish et al. 1998; Flynn and Chan 2001). This still leaves a staggering 90% of people infected with the tubercle bacillus who never develop disease. The inescapable conclusion, often overlooked, is that in the vast majority of cases host immunity appears more than adequate to hold the organism in check—indeed, so successful is it that many human tuberculous lesions are completely sterilised with time (reviewed in McKinney et al. 2001). Part of this success can be ascribed to the activated macrophage, a concept first enunciated and aptly shown in Lurie's classic studies over 60 years ago (Lurie 1939, 1942). Yet despite the insights provided by this work and that of earlier pioneers such as Metchnikoff, neither could have foreseen the complexity of the macrophage anti-tubercular arsenal, an elaborate system of defence that defies its phylogenetically primitive origins.

2
Beyond Metchnikoff: Emerging Complexity of the Macrophage Anti-Tubercular Arsenal

2.1
Sensory Logic of the Macrophage Against *Mtb*

2.1.1
Receptors for *Mtb* Recognition

Located at the interface of gaseous exchange between the outside world and respiring host, AMs appear well situated to sample the incoming repertoire of pathogen-specific motifs belonging to airborne infectious agents like *Mtb*. The necessity of AMs to discriminate not only between self and the external environment, but also between different micro-organisms, poses a serious challenge to innate pulmonary immunity, and one compounded by the high mutation rates of many inhaled pathogens. This challenge, however, has in part been met by an evolving set of receptors capable of recognising invariant microbial structures not found in higher eukaryotes. Janeway first proposed the term "pathogen-associated molecular patterns" (PAMPs) to embrace these conserved motifs and "pattern-recognition receptors" (PRRs) for the host apparatus which detects them (Janeway 1989). PAMPs share common features which allow efficient host recognition: prokaryotic specificity, invariance within a given microbial class (and hence detection by a limited number of germline-encoded PRRs), and lastly, obligate roles in microbial survival, such that alteration or loss would either be lethal or lead to a greatly reduced adaptive fitness. Microbial genes containing PAMPs would therefore not be subject to a high incidence of mutation and "escape mutants" less likely to be selected (Medzhitov 2001).

PAMPs include cell wall components such as yeast mannans, formylated bacterial peptides, trypanosome glycosyl phosphatidylinositol (GPI) linkages, and

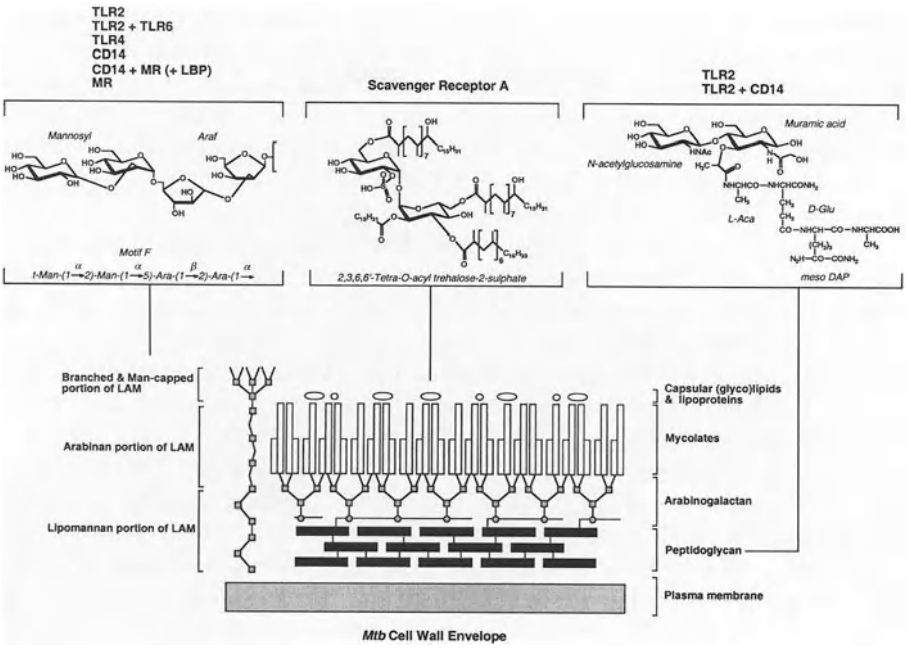

Fig. 1 Macrophage receptors for *Mtb* recognition

lipoteichoic acids and lipopolysaccharides (LPS) on gram-positive and gram-negative organisms, respectively (Aderem and Ulevitch 2000; Medzhitov 2001). Likewise, recognition of *Mtb* by macrophages takes advantage of the unique lipid-rich outer envelope found in all *Mycobacterium* spp., thus satisfying the first two PAMP criteria. That this unusually complex cell wall contributes to the evolutionary fitness of *Mtb*—thereby complying with the third PAMP criterion—is based on a remarkable ability to resist chemical, physical and chemotherapeutic stresses, to withstand macrophage killing mechanisms in order to replicate intracellularly, and to act as a potent adjuvant, the latter of which may elicit immunopathology as an to aid further bronchial spread (Dannenberg and Rook 1994; McKinney et al. 1998).

Largely due to the efforts of Brennan and co-workers, the surface chemical composition of virulent *Mtb* has been well characterised (reviewed in Baulard et al. 1999), enabling some of the molecular signatures buried within to be defined. Prominent among the glycolipids non-covalently attached to the peptidoglycan–arabinogalactan–mycolate scaffold is the mannose-capped lipoarabinomannan (ManLAM) (Fig. 1). ManLAM is recognised in non-opsonised form by several human and mouse macrophage PRRs: Toll-like receptors (TLRs), mannose (C-type lectin) receptors (MRs), and CD14 (Pugin et al. 1994; Schlessinger et al. 1994; Schlessinger et al. 1996; Prigozy et al. 1997; Means et al. 1999; Underhill et al. 1999b). Within this overlap, as well as for other *Mtb* cell wall components, a finer specificity of recognition has begun to emerge. For example, TLRs 2 and 4

both respond to ManLAM, whereas only TLR2 recognises AraLAM from rapidly growing mycobacteria (Means et al. 1999; Underhill et al. 1999b; Means et al. 2001). Class A scavenger receptors ignore both mannosylated and arabinosyl LAM moieties yet scrutinise trehalose 2-sulphate derivatives (Ernst 1998). TLRs 2 and 6 scan peptidoglycan, but only TLR2 detects lipopeptides, phosphatidylinositol mannosides or the *Mtb* 19-kDa lipoprotein (Brightbill et al. 1999; Underhill et al. 1999b; Ozinsky et al. 2000; Jones et al. 2001).

Receptor co-operativity confers another level of sophistication in which discrete signalling modules are assembled to activate certain macrophage responses and direct effector localisation. TLRs 2 and 6 physically associate in order to recognise peptidoglycan and signal tumour necrosis factor alpha (TNF) production, while TLR4 can elicit TNF as a homodimer (Ozinsky et al. 2000). Induction of this cytokine, as well as interleukins (IL)-6 and -12, appears reliant on MyD88 binding to the cytoplasmic tail of each of these TLRs, whereas nitric oxide (NO) secretion does not (Brightbill et al. 1999; Kawai et al. 1999; Underhill et al. 1999a,b; Means et al. 2001). Combinatorial effects also are evident for CD14, a soluble or GPI-linked membrane-bound receptor which lacks a cytoplasmic tail and therefore requires binding partners to help transduce its intracellular signal. Here, CD14 can complex with TLR2 to bind peptidoglycan (Yang et al. 1999b) or with MRs on human macrophages to recognise LAM (Bernardo et al. 1998), a detection couplet which may acquire further sensitivity via the interaction of LAM with the LPS-binding protein (Savedra et al. 1996). Once associated with a particular PRR, the particle-receptor complex could have several destinational fates. Binding of LAM to surface MRs leads to lysosomal delivery (Prigozy et al. 1997) and TLR2, singly or in combination with TLR6, is recruited to phagosomes where it may survey the contents as part of an ongoing homeostatic mechanism (Underhill et al. 1999a; Ozinsky et al. 2000).

2.1.2
Receptors for *Mtb* Uptake

Metchnikoff invoked the term, *fresszellen* (or devouring cells), in his manuscript on *Daphnia* to describe the gustatory activities of primitive mesoderm (Metchnikoff 1884). Having tasted the lipid-laden surface of *Mtb*, macrophages subsequently employ a variety of phagocytic receptors including PRRs to ingest the organism. Unlike pinocytosis, which involves passive solute uptake, or receptor-mediated endocytosis, which enlists clathrin-coated pits for receipt of smaller molecules, phagocytosis uses both receptor diversity to increase its particle range (>0.5 μm) and actin-driven cytoskeletal remodelling to increase the rate of internalisation (Aderem and Underhill 1999). For *Mtb*, a particle 1–4 μm in length and 0.3–0.6 μm in diameter, this task extends primarily to the complement (CR) and Fc (FcR) receptor families, although some assistance may be rendered by CD43 and fibronectin as well (Fratazzi et al. 2000; Pasula et al. 2002). These receptors enable macrophages to capture mycobacteria via complement or antibody (Ab) fixation, respectively. They also indirectly bind different

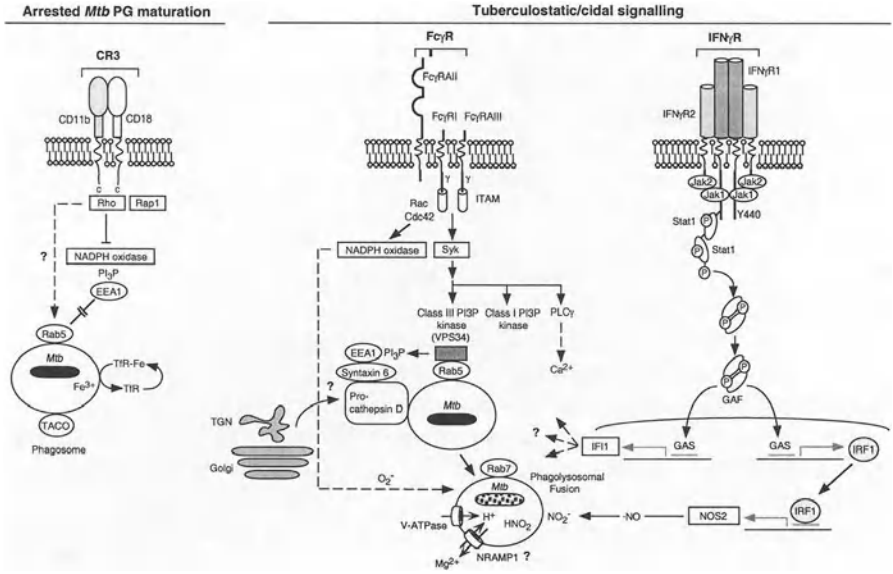

Fig. 2 Receptor-dependent fate of *Mtb* in macrophages

solvent structures on *Mtb*: C3b, C3bi, and C4b cleavage products (recognised by CRs 1, 3 and 4) attach lipid/carbohydrate moieties while Ig domains ensure a (glyco)protein template contact (Ernst 1998).

A role for CRs was first suggested by experiments in which small amounts of fresh (complement-replete) autologous non-immune serum led to enhanced phagocytosis by human monocytes (Schlesinger et al. 1990). Opsonic entry of virulent *Mtb* indicated that as little as 1% serum was sufficient for maximal enhancement (Schlesinger et al. 1990). This percentage is found in the lungs of healthy non-smokers for C3bi and C4b, primarily secreted by AMs, type II epithelia and fibroblasts (Reynolds and Newball 1974; Hill 1993). Human AMs appear particularly reliant on CR4 to mediate mycobacterial uptake (Hirsch et al. 1994; Zaffran et al. 1998) while in human monocytes, *Mtb* internalisation may be blocked by as much as 80% using monoclonal antibodies against CR3 (Schlesinger et al. 1990). Further evidence for CR3 involvement comes from recent studies of CD11b-deficient mice, since this integrin serves as the α-chain in the CD11b/CD18 heterodimer (Fig. 2). Resident macrophages from CD11b$^{-/-}$/$^{-}$ mice exhibit 40%–50% lower levels of serum-mediated *Mtb* uptake and a 50%–60% reduction in non-opsonic binding (Melo et al. 2000), the latter normally mediated via capsular polysaccharides interacting with the CD11b-glucan binding site (Cywes et al. 1997). Despite the reduced internalisation, CD11b$^{-/-}$ mice were no more or less susceptible to TB, like their CR3$^{-/-}$ counterparts (Hu et al. 2000; Melo et al. 2000). Clearly other receptors help control growth of the pathogen.

Engagement of FcRs may lead to better restriction of *Mtb* growth as it usually triggers an oxidative burst, while ligation of CRs does not (Douvas et al. 1986; Kobzik et al. 1990). FcR participation also depends on the maturational state of the cell; it is all but absent in human monocytes (Schlesinger et al. 1990) yet operative in terminally differentiated macrophages (Malik et al. 2000, 2001). Abundant expression of FcγRsI, IIA and IIIA on adult human AMs together with elevated pulmonary IgG levels during TB also raises the likelihood that FcR-mediated uptake of *Mtb* occurs in vivo (Naegel et al. 1984; Fukushima et al. 1991; Sharma et al. 1992). Such uptake leads to a rise in cytosolic Ca^{2+} which promotes phagosome maturation (Armstrong and Hart 1975; Malik et al. 2000), nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) oxidase assembly (Zhou et al. 1997; Melendez et al. 2001), and, at least in the case of human macrophages, killing (Malik et al. 2000) (Fig. 2).

The above studies indicate that the route of *Mtb* entry can influence the organism's fate, and raise the issue of whether *Mtb* internalisation is solely an active host process or assisted by the pathogen. The *Mtb mce1* gene, for example, heterologously conferred invasive properties on *Escherichia coli* for HeLa cells (Arrunda et al. 1993) although its function in *Mtb* awaits confirmation. Preferential uptake by CRs and avoidance of an oxidative burst may be mediated by mycobacteria through salvage of C2a to assemble a C3 convertase on its surface, resulting in cleavage and deposition of C3bi (Schorey et al. 1997). Others have suggested that *Mtb* may selectively use cholesterol-rich caveolae or lipid rafts as portals of entry (Pieters 2001). Mycobacterial products assigned such a function, however, have yet to be identified.

2.2
Cognitive Logic of the Macrophage Against *Mtb*

2.2.1
Intracellular Sorting and the Conduits for Antigen Presentation and Elimination

Almost immediately after uptake, macrophages attempt to dispose of *Mtb* via a series of vesicular transportation pathways leading to lysosomal degradation. These vesicles were among the most striking ultrastructural features of TB granulomata noted by early investigators (Dumont and Sheldon 1965). We now realise they also help establish memory of the encounter through their intersection with major histocompatibility complex (MHC) and CD1 loading compartments.

Podal closure around bacilli and F actin depolymerisation give rise to newly formed phagosomes (PGs) displaying a composition similar to the plasma membrane from which they originate. However, many of the plasma membrane proteins (including FcγRII and MR; Muller et al. 1983) are lost within 3–5 min after PG formation (Pitt et al. 1992; Oh and Swanson 1996). Others, like MHC class II, transferrin receptor, cellubrevin/VAMP3, soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP)-23 and syntaxins 3 and 4, remain from

20 min to 72 h on the mycobacterial PG surface (Clemens and Horwitz 1995; Fratti et al. 2000). So does ARF6 (Fratti et al. 2000), which in addition to its involvement in actin rearrangement, recycles endosomal vesicles to the plasma membrane (Al-awar et al. 2000). This protein profile, together with the fact that nascent PGs become accessible to plasma membrane-derived glycosphingolipids and exogenously added tracers like transferrin as early as 5 min after *Mtb* uptake, all suggest an interaction with the recycling endosome network (Clemens and Horwitz 1996; Russell et al. 1996). Fusion with early endosomes similarly results in delivery of the small monomeric guanosine 5′ triphosphatase (GTPase) rab5 (Clemens et al. 2000b; Fratti et al. 2000), considered the critical timer for endosomal docking and fusion (reviewed in Zerial and Mcbride 2001).

It is at this juncture that the mycobacterial PG either proceeds toward a hydrolytically competent phagolysosome (PL) or undergoes maturational arrest (Via et al. 1997; Fratti et al. 2000). The outcome appears largely dictated by the route of *Mtb* entry and activation status of the host cell (Fig. 2). Prevention of PL fusion was first observed by Hart and colleagues using unstimulated macrophages in which viable *Mtb* failed to co-localise with acid phosphatase-rich lysosomes (Armstrong and Hart 1971; Armstrong and Hart 1975). In contrast, *Mtb* that had been coated with Ab or rendered non-viable by irradiation were delivered to lysosomes. Work conducted 25–30 years later suggests the block resides at the level of rab5 and one of its effectors, the early endosome autoantigen 1 (EEA1) (Fratti et al. 2001). EEA1, a tethering molecule that normally couples vesicle docking with SNAP receptor (SNARE) priming, is excluded from unopsonised mycobacterial PGs while present on those encircling latex beads. Moreover, EEA1 is recruited to the latter PGs within 10 min of uptake via its FYVE-domain association with phosphatidyl inositol 3-phosphate (PI_3P), a product generated within the PG membrane (Ellson et al. 2001) by the class III PI_3P kinase VPS34, which directly binds rab5 (Fratti et al. 2001; Vieira et al. 2001). Antibody neutralisation or use of cells deficient for VPS34 have both established the necessity of this kinase for PL development.

The specific involvement of PI_3P could also link Ab-opsonised *Mtb* with the PL maturation initially seen by Armstrong and Hart (1975), an uptake presumably mediated via FcγRs since F'ab-coated *Mtb* does not lead to vacuole acidification (Malik et al. 2000). Clustered FcRs provide docking sites for Syk kinase which has been implicated in lysosomal targeting (Bonnerot et al. 1998). Syk forms the nexus for at least three signalling pathways: (1) class I PI3 kinase, which generates $PI(3,4,5)P_3$ for amphiphysin IIm, dynamin, ARF6 and Rab 11 recruitment, leading to early events such as pseudopod extension and PG closure; (2) PLCγ activation and $PI(1,4,5)P_3$, diacylglycerol and Ca^{2+} mobilisation; and (3) class III PI3 kinase, which generates PI(3)P for EEA1-mediated Rab5/SNARE association and the next stage of PG-lysosome fusion (Crowley et al. 1997; Aderem and Underhill 1999; Greenberg and Grinstein 2002). This stage, characteristically seen in macrophages activated with IFN-γ plus LPS, coincides with a loss of rab5 from the PG (Via et al. 1997) and inaccessibility to transferrin (Schaible et al. 1998). The latter effect may help limit the supply of iron

chelated by *Mtb* siderophores for microbial growth (Gobin and Horwitz 1996; De Voss et al. 2000). By 4 h post-uptake, the *Mtb* PL within activated macrophages has acquired the late endosomal marker rab7, lysosomal integral membrane proteins (LGPS or LAMPS), and vacuolar H^+ ATPase (V-ATPase) pump subunits (Xu et al. 1994; Fratti et al. 2000), the last responsible for a drop in lumenal pH from ~6.5 to 5.2 (Schaible et al. 1998). This environment allows for the processing of immature cathepsins and activation of acid phosphatases (Armstrong and Hart 1975; Ullrich et al. 1999) following their likely arrival from the trans-golgi network through the aid of EEA1 binding to syntaxin 6-containing vesicles (Fig. 2; Simonsen et al. 1999; Fratti et al. 2002). Such changes are thought to precede a decrease in mycobacterial viability (Schaible et al. 1998).

How the transition from PG to PL occurs is still unclear. It could enlist repeated, transient vesicular transfer between PGs and late endosomes/lysosomes (a "kiss-and-run" model; Desjardins 1995) or undergo direct fusion with these organelles to form a hybrid compartment (Mullock et al. 1998). The recent discovery of the Rab7 effector RILP (Rab7-interacting lysosomal protein; Cantalupo et al. 2001) has shed some light on the directional nature of PL trafficking. RILP recruits dynein–dynactin motor complexes to Rab7-containing late endosomes/lysosomes, resulting in vesicular transport to the minus end of microtubules rather than towards the macrophage periphery (Jordens et al. 2001). Whether the Rab7-RILP complex is important for polarised maturation of *Mtb* PLs awaits enquiry, although in infected fibroblasts Rab7 appears dispensable (Clemens et al. 2000b). SNARE proteins such as syntaxin 7 and VAMP7, both required for late endosome–lysosome fusion in human AMs (Ward et al. 2000), or the recently cloned Vam6p (Caplan et al. 2001), are among other candidates for this role.

Not all endosomal trafficking proceeds terminally towards lysosomes, which can themselves recycle some of their cargo to the plasma membrane via newly described exosomes (Denzer et al. 2000). For *Mtb*-derived antigens to be presented at the cell surface, loading and sorting must entail retrograde transport. The compartment in which antigens are processed and exported for presentation is very much dependent on their composition. *Mtb N*-formyl methionine peptides, for example, associate with H2-M3 MHC class 1b in the endoplasmic reticulum (Chun et al. 2001), while Ag85 is bound to $I\text{-}A^b$ in specialised MHC class II compartments (MIICs) (Ramachandra et al. 2001). In humans the latter are enriched for LAMPs 1–3, acid hydrolases, and HLA-DM and HLA-DO which regulate peptide-MHC class II assembly (Geuze 1998). Recycling of antigen-MHC complexes to the plasma membrane is also rapid, with Ag85-bound $I\text{-}A^b$ being detected as soon 20 min after uptake (Ramachandra et al. 2001). *Mtb* (glyco)lipids encounter a different set of antigen-presenting molecules: the MHC class I-related CD1 family (Moody and Porcelli 2001). CD1 isoforms are distributed along the endocytic highway; on the plasma membrane/early endosomes (CD1a), early to late endosomes (CD1c), late endosomes/lysosomes (CD1d/CD1e) and MIICs (CD1b). There is thus ample opportunity to sample *Mtb* lipids like LAM, mycolic acids, polyisoprenols, phosphatidyl-inositol man-

nosides, and lysocardiolipin detected within the macrophage endosomal network (Beatty et al. 2000; Ficsher et al. 2001). Once presented at the surface, human T cells recognising these *Mtb*-derived glycolipids produce cytokines such as IFN-γ (Stenger et al. 1997) to further incite macrophage antimicrobial responses.

2.2.2 Receptors for Activation

Discovery of IFN-γ as the principal macrophage-activating cytokine (Nathan et al. 1983; Schreiber et al. 1983; Nathan et al. 1984) soon led to reports of its effectiveness in curtailing *Mtb* replication (Rook et al. 1986a; Flesch and Kaufmann 1987). It also opened the door to a molecular description of how phagocytes receive and integrate such signals from cell surface to nucleus (Bach et al. 1997).

When both chains of the IFN-γreceptor (IFNGR) bind cytokine, they dimerise, allowing Janus kinases (Jaks) 1 and 2 attached to their cytoplasmic tails to phosphorylate each other. Jaks can further phosphorylate IFNGR1 via the classical pathway (Ramana et al. 2002) to then create docking sites for STAT1 (signal transducer and activator of transcription-1). These latent transcription factors pair to form gamma-activated factors (GAFs) and translocate to the nucleus via the importin-α5 complex (McBride et al. 2002), where they bind IFN-γ activation site (GAS) elements in the promoters of IFN-γ-inducible genes. All this transpires within 5–20 min of receptor engagement (Bach et al. 1997; Darnell 1997). It also initiates waves of secondary transcription via target genes like interferon regulatory factor-1 (IRF-1) and IFN consensus sequence binding protein (ICSBP/IRF-8), which themselves enhance the later expression of macrophage genes such as inducible nitric oxide synthase (NOS2) and FcγR1 (Contursi et al. 2000). Autocrine stimulation of this pathway during TB is also likely, since human AMs are a significant source of IFN-γ (Fenton et al. 1997).

That IFN-γ and IFNGR signalling components are critical for protection against TB is based on several lines of evidence. First, latently infected individuals or exposed asymptomatic household contacts exhibit higher levels of IFN-γ-secreting bronchoalveolar lavage (BAL) or peripheral blood mononuclear cells (PBMC) than do patients with culture-positive pulmonary TB (Schwander et al. 2000; Pathan et al. 2001). Thus IFN-γ expression may correlate with resistance to developing disease. Second, loss-of-function mutations lead to TB susceptibility, as most dramatically demonstrated in IFN-$\gamma^{-/-}$ (Cooper et al. 1993; Flynn et al. 1993), IFN-γR1$^{-/-}$ or STAT1$^{-/-}$ mice (J. MacMicking, unpublished results). The same could apply to humans; hypofunctional and nonfunctional (null) mutations in IFNGRs and STAT1 all lead to greatly impaired anti-mycobacterial immunity, as do mutations in the p40 subunit of the IFN-γ-inducing cytokine, IL-12, or the IL-12 receptor chain (Casanova and Abel 2002). Human macrophages may also be made refractory to IFN-γ signalling by *Mtb* itself, which can interfere with STAT1 binding its transcriptional coactivators CBP and p300 (Ting et al. 1999). Lastly, transgenic IFN-γ reconstitution in the lungs of nullizygous

mice had significant ameliorative effects (Collins and Kaufmann 2001), while provision of recombinant IFN-γ alleviated bacterial burdens in patients who were non-responsive to antimicrobials and conferred bactericidal activity on human macrophages in culture (Condos et al. 1997; Bonecini-Almeida et al. 1998).

The effectiveness of IFN-γ in programming macrophages for *Mtb* clearance is augmented by the action of another cytokine, TNF (Chan et al. 1992). Such synergy is a recurring theme in cytokine biology and provides several advantages: (1) increased diversity of the responding host gene repertoire due to alternate transcription factor usage; (2) higher rates of synthesis for those genes with promoters possessing binding sites for both sets of transcription factors; and (3) a lower signalling threshold brought about by shared pre-existing components. The latter is especially germane given the relative paucity of IFNGRs (4,000–12,000/cell) on the macrophage surface (Pace et al. 1983; Finbloom et al. 1985). Examples of the other two benefits are readily appreciated if one examines NOS2 induction. This 260-kDa flavoenzyme requires each of its monomers to be transcribed via STAT1/IRF1-dependent signalling, yet induction is profoundly enhanced by a second signal, such as nuclear factor (NF)-κB originating from TNF or LPS (or LAM) stimulation (MacMicking et al. 1997b; Chan et al. 2001). Moreover, TNF elicits GTP cyclohydrolase 1, which furnishes an essential cofactor (BH_4) for NOS2 catalysis, while NF-κB signals the argininosuccinate synthetase and cationic amino acid transporter 2 genes needed to regenerate and import L-arginine, a NOS2 substrate (Bogdan 2001).

Not only are there co-operative effects between TNF and IFN-γ but also between individual TNF receptors 1 (TNFR1) and 2 (TNFR2); their co-ligation in the presence of IFN-γ leads to a more sustained NO production than with either alone (Riches et al. 1998). This is consistent with a "ligand passing" model in which at low TNF concentrations, the higher affinity and more rapid K_a/K_d of TNFR2 ensures ligand capture for TNFR1 as part of a binary complex (Pickard et al. 1997). TNFR1 then preferentially recruits TNF receptor-associated factor 2 (TRAF2) by virtue of its higher affinity for the TNFR1-associated death domain protein (TRADD) (Baud and Karin 2001). NOS2 induction thereby benefits from the coupling of a stronger intracellular signal issued by TNFR1 (Riches et al. 1998) with the extracellular ligand sensitivity provided by TNFR2.

In mice lacking TNFR1 or TNF, or receiving inhibitory TNFR fusion proteins, *Mtb* grows unabated until death of the host within weeks after infection compared with months for untreated controls (Adams et al. 1995; Flynn et al. 1995; Bean et al. 1999). Some of this marked susceptibility was attributed to diminished macrophage NO production within the first 10 days (Flynn et al. 1995). In humans, administration of infliximab (an anti-TNF mAb) as part of the treatment for rheumatoid and Crohn's disease led to prompt recrudescence of TB in 70 patients who were latently infected (Keane et al. 2001). Natural inhibitory forms of soluble TNFRs (sTNFRI and II) comprising just the extracellular domain could also diminish TNF levels, especially since these are greatly elevated in the serum of active TB patients versus healthy non-TB cohorts, TB contacts

or those on antituberculous therapy (Juffermans et al. 1998). Heightened release of sTNFRs has similarly been seen in vitro for *Mtb*-infected AMs (Balcewitz-Sablibska et al. 1998). Each report suggests the TNF/TNFR axis otherwise protects against TB in the human population.

Secreted lymphotoxin (LT-α_3, or TNF-β) serves as another ligand for both TNFRs, and exhibits affinity constants commensurate with those of soluble TNF (Loetscher et al. 1991). Yet unlike TNFR1$^{-/-}$ or TNF$^{-/-}$ mice, LT$\alpha^{-/-}$ chimeras showed uncompromised NOS2 induction despite being equally susceptible to TB (Roach et al. 2001). This highlights two points. First, there exist mechanisms besides NO for *Mtb* containment, notably structural integrity of the granuloma (Roach et al. 2001). Second, lung macrophages probably rely more heavily on autocrine TNFR signalling to induce NO synthesis, since TNF (primarily a monokine) substitutes in the absence of LT (a lymphokine) but not vice versa.

TLR2 and members of the P2 purinergic receptor family have recently been added to the list of receptors involved in activating the macrophage anti-tubercular arsenal. Specific TLR2 engagement using the *Mtb* 19-kDa lipoprotein elicits mouse macrophage NOS2 for restriction of *Mtb* growth, while in human AMs a novel NOS2-, TNF-independent pathway apparently exists (Thoma-Uszynski et al. 2001). P2 purinergic receptor subtypes are likewise engaged in differential fashion: $P2X_7$ activates cytosolic Ca^{2+} release and phospholipase D for maturation of *Mtb* phagosomes in human macrophages (Kusner and Adams 2000; Kusner and Barton 2001), whereas subtypes other than $P2X_7$ stimulate *Mtb* killing via NOS2 and possibly reactive oxygen intermediates (ROI) in the mouse (Sikora et al. 1999).

Though not considered a macrophage-activating cytokine in the classical sense, vitamin D_3 [25(OH)$_2$D3] and its ligand-binding receptor (VDR) deserve special mention in the context of TB. Calcitriol [1,25-(OH)$_2$D3] was shown in early studies to be crucial in aiding the tuberculostatic action of IFN-γ in human macrophages (Rook et al. 1986b; Crowle et al. 1987). This bioactive D3 metabolite is synthesised from 25(OH)$_2$D3 by 25(OH)$_2$D3-1 α-hydroxylase and degraded by 24-hydroxylase, enzymes respectively stimulated and inhibited by IFN-γ (Adams and Gacad 1985; Koeffler et al. 1985; Reichel et al. 1987). Calcitriol in turn promotes STAT1-VDR association and the transactivation of IFN-γ-inducible genes (Vidal et al. 2002), suggesting an involvement beyond its well-known effects on macrophage differentiation (Hmama et al. 1999). It is therefore not surprising that some correlation between VDR affinity and the anti-tubercular efficacy of individual metabolites was noted in the initial studies. More recent experiments suggest non-orthodox VDR signalling could account for these observations, because D_3 activates PI3 kinase plus an oxidative burst in PBMCs and NOS2 in human and mouse myelomonocytic cell lines (Rockett et al. 1998; Sly et al. 2001). Moreover, D_3 may contribute to protection in vivo: VDR polymorphisms and TB susceptibility appear to be linked in certain African and Asian populations (Bellamy et al. 1999; Wilkinson et al. 2000).

2.2.3
Deployment of Macrophage Tuberculostatic and Cidal Mechanisms

Nathan and colleagues developed stringent criteria to assess whether a given macrophage effector mechanism operates against a particular pathogen (MacMicking et al. 1997b). They include: (1) correlation of host gene expression with resistance; (2) conferral of direct antimicrobial activity by the host gene or its product (see e.g. Karupiah et al. 1993); (3) loss of host gene function leading to enhanced microbial growth in vitro (via pharmacologic inhibition, RNA interference) or disease exacerbation in vivo (using natural and engineered host mutants, or drug ablation); and (4) evolution of microbial genes resistant to the mechanism in question. The latter would infer its existence through selective pressure. Based on all four criteria, reactive nitrogen intermediates (RNI) arising from the generation of NO by NOS2 emerge as the primary, albeit imperfect, candidates against *Mtb*. Two others, ROI and PL fusion, await further clarification, while more recently a fourth, the β-defensins, has yet to be demonstrated as a natural product of human macrophages (Kisich et al. 2001).

The case for RNI in vitro is compelling. Direct exposure of *Mtb* to as little as 90 ppm of NO gas —approaching the exhaled concentrations found for some pulmonary TB patients (Wang et al. 1998) —kills more than 99% of plated organisms (Long et al. 1999). Unstable derivatives such as nitrogen dioxide (NO_2) appear even more potent (Yu et al. 1999), while the acidified milieu of the macrophage PL may help retrieve additional NO equivalents from stably oxidised forms (e.g. NO_2^-) by protonation to HNO_2 and subsequent dismutation (MacMicking et al. 1997b). Indeed, acidified $NaNO_2$ was as one of the earliest compounds employed in demonstrating a role for NO in direct killing of *Mtb* (Chan et al. 1992). Susceptibility of various *Mtb* strains to acidified $NaNO_2$ correlated inversely with their virulence in guinea-pigs (O'Brien et al. 1994) as well as suggesting the presence of detoxifying pathways on behalf of the pathogen (Rhoades and Orme 1997).

Causal relationships between macrophage NOS2 expression and loss of *Mtb* viability confirm results found in cell-free systems. *Mtb* inhibition in Bcg^r and Bcg^s macrophages correlates with NO production (Arias et al. 1997), while activated murine macrophages treated with NOS inhibitors (Denis 1991a; Chan et al. 1992) or in which the *NOS2* locus has been genetically disrupted (Bodnar et al. 2001; Erht et al. 2001) exhibit little tuberculocidal activity. In human macrophages, eliciting NOS2 via cytokines and/or microbial products appears more complex, akin to human lung epithelia where at least three independent stimuli—IFN-γ, TNF, and IL-1—are required to produce a relatively weak transcriptional response due to STAT1 and NF-κB acting at some distance (~5–8 kbp) from the start site (Ganster et al. 2001). Restricted NOS2 cofactor availability, especially BH_4, could also contribute to the generally lower RNI production of human versus rodent cells (Berholet et al. 1999). Nonetheless, recent evidence shows that macrophages taken from the inflamed lungs or peripheral blood of TB patients, or AMs from healthy donors subsequently infected in vitro, can ex-

press NOS2 and produce mycobactericidal amounts of NO (refer to Dlugovitzky et al. 2000; Nathan and Shiloh 2000; Means et al. 2001; Wang et al. 2001). Moreover, where examined, anti-tubercular effects were blocked by NOS inhibitors.

A case for RNI-dependent protection extends to the intact host. Macrophage NOS2 was present in TB granulomas of humans (Fachetti et al. 1999) and resistant wild-type mice (MacMicking et al. 1997a; Mogues et al. 2001); the latter were rendered susceptible with specific NOS2 inhibitors like N^6-(1-iminoethyl)-L-lysine (MacMicking et al. 1997a) and drugs of lower isoform specificity such as aminoguanidine and *N*-methyl-L-arginine (Chan et al. 1995; Flynn et al. 1998). More convincing evidence is provided by $NOS2^{-/-}$ mice, which are extremely sensitive to *Mtb* infection (MacMicking et al. 1997a; Mogues et al. 2001; Scanga et al. 2001). The same is true of other gene-deficient mice (IFN-$\gamma^{-/-}$, $TNFR1^{-/-}$ or $TNF^{-/-}$) as well as glucocorticoid-immunosuppressed and malnourished animals exhibiting secondary defects in macrophage NOS2 expression (reviewed in Nathan and Shiloh 2000). Lastly, the presence of RNI resistance genes may indirectly suggest a role for this pathway in the host response to TB. *NoxR1*, *noxR3*, alkyl hydroperoxide reductase (*ahpC*), peptidyl methionine sulphoxide reductase (*msrA*), dihydrolipoamide dehydrogenase (*Lpd*) and dihydrolipoamide succinyl-transferase (*SucB*) are each posited as *Mtb* RNI resistance genes (Nathan and Shiloh 2000; St. John et al. 2001; Bryk et al. 2002), although chromosomal inactivation has yet to corroborate these claims (Stewart et al. 2000; Springer et al. 2001). Disruption of the oligopeptide permease operon (*oppBCA*), which can transport NO-thiol adducts across the mycobacterial cytoplasmic membrane, confers resistance to *S*-nitrosoglutathione and suggests that NO could be more effective as a congener (Green et al. 2000).

Virulent *Mtb* grown in culture or in murine macrophages are relatively resistant to the effects of ROI, and host cells either treated with ROI scavengers or deficient in the production of superoxide (O_2^-) still managed to restrict *Mtb* replication (Flesch and Kaufmann 1987; O'brien and Andrew 1991; Chan et al. 1992; Adams et al. 1997). Likewise, an oxidative burst triggered by mycobacterial agonists in IFN-γ-primed $NOS2^{-/-}$ macrophages still failed to inhibit microbial growth (MacMicking et al. 1995; Erht et al. 2001). Mice with targeted disruptions in either $gp47^{phox}$ or $gp91^{phox}$ subunits of the periplasmic NADPH oxidase responsible for O_2^- synthesis allow very modest increases in lung bacterial burdens versus wild-type controls (Adams et al. 1997; Cooper et al. 2000). Moreover, humans with crippling mutations in any one of four NADPH oxidase subunits (collectively referred to as chronic granulomatous disease; CGD) do not appear especially vulnerable to infections by mycobacteria (Segal et al. 2000), although anti-tubercular immunity may be impaired in some patients (Lau et al. 1998).

A major reason for the limited potency of ROI against *Mtb* is that the bacterium robustly expresses ROI-detoxifying enzymes (e.g. catalase, superoxide dismutase, peroxiredoxins) both within and outside activated human macrophages (Andersen et al. 1991; Mariani et al. 2000). Such enzymes could also serve to counteract oxidative species arising as by-products of bacterial respiration. Ad-

ditionally they may work in concert with RNI neutralising pathways to limit the production of compound intermediates such as peroxynitrite ($ONOO^-$), a powerful oxidant that can decompose under acidic conditions to NO_2 and the hydroxyl radical ($OH^·$). Virulent strains of *Mtb*, for example, appear to be more resistant to $ONOO^-$ than non-pathogenic strains (Yu et al. 1999), reminiscent of studies examining sensitivities to hydrogen peroxide (H_2O_2) (Jackett et al. 1978; Laochumroonvorapong et al. 1997). Another reason could include the fact that catalase and peroxiredoxins are also directly effective at scavenging RNI (reviewed in Nathan and Shiloh 2000). Both scenarios may be examined using mice that are doubly deficient for NOS2 and phox (Shiloh et al. 1999) together with the respective *Mtb* mutants, a genetic approach similar to that already employed for the *Mtb sodC* gene (Piddington et al. 2001).

The antimicrobial actions of both RNI and ROI are potentiated by low pH and iron (usually Fe[III]), the latter incorporated into dinitrosyl-iron complexes as well as helping drive the Haber-Weiss reaction for $OH^·$ formation. The usefulness of acid in host defence was first suggested by Metchnikoff (1893) and gained credence from two early observations: that phagocytes acidify their PLs (Rous 1925) and that achlorhydric hosts permit bacterial colonisation of the stomach (Gianella et al. 1973). In the case of mycobacteria, the macrophage PL acidifies to pH ~4.5–5.0 following cytokine stimulation, irrespective of whether the agonist is given before or after bacterial uptake (Schaible et al. 1998; J. MacMicking, unpublished). Acidification coincides with the delivery of V-ATPases, enables processing of lysosomal hydrolases, and correlates with diminished *Mtb* growth (Schaible et al. 1998; Gomes et al. 1999; Ullrich et al. 1999). Conversely, agents that specifically inhibit the ATPase (e.g. omeprezole) allow increased *Mtb* replication in human AMs (Suzuki et al. 2000). As yet there is no reliable information on whether such mechanisms operate in vivo, a void difficult to fill, since natural and engineered ATPase mutants, or pharmacologic manipulation within the intact host, would surely have widespread homeostatic consequences for the cell. Perhaps gain-of-function studies involving forced PL maturation on an RNI/ROI-deficient background may yield information about the requirement for acid per se.

The last point is especially relevant for *Mtb* since it was originally shown to be unique among other *Mycobacteria* spp. in its sensitivity to pH less than 6.5, with a marked attenuation for growth at pH 5.0 (Chapman and Bernard 1962). Later studies, however, found little difference in viability between pH 4.5–7.0 (Jackett et al. 1978; Chan et al. 1992). An explanation for this discordance may rest with the use of complex media varying in divalent cation concentration. When grown at lower pH under restricted Mg^{2+} conditions, for example, *Mtb* fares much less well (Piddington et al. 2000). These conditions could also mimic that found in the host: an *Mtb* mutant lacking the *mgtC* (magnesium transporter) gene replicates poorly at low pH and Mg^{2+} concentration, and its growth is highly attenuated in human macrophages and the lungs of mice (Buchmeier et al. 2000).

Mtb similarly imports other divalent cations—Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}—via expression of *Mramp*, a pH-dependent transporter which functions optimally at pH 5.5–6.5 and is upregulated under axenic culture at low pH as well as in human macrophages (Agaroff et al. 1999). In this respect it may compete with its mammalian homologue, natural resistance-associated macrophage protein 1 (NRAMP1, or SLC11A1), a H^+/divalent cation antiporter located at the phagosomal membrane (Jabado et al. 2000). A caveat to this relationship is that while NRAMP1's role in protecting the host against mycobacteria other than *Mtb* is well established, its deletion has no effect on TB susceptibility in mice (North et al. 1999). The case in humans is less clear: a lack of association between TB susceptibility and NRAMP1 status was noted for Brazilian populations (Blackwell et al. 1997) while *NRAMP1* polymorphisms in West Africans, Japanese and Koreans may carry an increased risk (Bellamy et al. 1998; Gao et al. 2000; Ryu et al. 2000).

Realisation that *Mtb* could tolerate an acidified, nutrient-poor environment moves understanding beyond the current preoccupation with models of how the bacterium blocks PL fusion. This latter effect, perhaps involving (1) bacterial ammonia generation via urease or sulphatide (Gordon et al. 1980; Goren et al. 1976) or (2) a host tryptophan aspartate-containing coat protein (TACO, or coronin-1; Ferrari et al. 1999), appears relevant only in macrophages that have not been activated (Via et al. 1997; Schaible et al. 1998). Of course, strategies for preventing activation are likely to exist: eliciting deactivating cytokines (e.g. IL-10, transforming growth factor-beta; Murray 1999) or evading antigen presentation altogether (Pancholi et al. 1993) are two such examples. Nonetheless, more attention should perhaps focus on examining bacterial adaptations within a hostile cell and to the combined rather than isolated actions of macrophage tuberculostatic/cidal pathways. Equally, consideration could be given to higher-order structure, where substratum, stromal- and T-cell contact within granulomas provide additional stimuli and where anatomical specialisations (e.g. to low oxygen tension) may be paramount.

2.3 Systems Logic of the Macrophage Against *Mtb*

2.3.1 Macrophage–Lymphocyte Networks: The Role of Granulomas

In humans, tuberculoid granulomas occupy not only the lungs, but anatomical sites as diverse as the larynx, palate, nasal septum, submaxillary and tracheobronchial lymph nodes, spine, bone marrow, genitourinary tract and peritoneal lining (Iseman 2000). Events leading to such widespread dissemination were chronicled in the preantibiotic era by Canetti (1955). From histobacteriologic examination of 30 cadavers, he suggested the following sequelae: a (pre)exudative stage dominated by mononuclear infiltrates and small numbers of AFB; walling off and caseous necrosis at the centre of the granuloma with progres-

sively fewer AFB; lesion resolution by fibrin deposition, sclerosis and calcification accompanied by disappearance of AFB. Alternatively, there could be caseum liquefaction and focal reappearance of AFB in the liquefied areas, the latter of which may rupture into a bronchus with discharge rendering the patient infectious.

The cellular composition and structure of the granuloma appears critical to whether TB lesions regress or become "open". Poorly organised granulomas, as seen in IFN-$\gamma^{-/-}$, TNF$^{-/-}$ and intercellular adhesion molecule (ICAM)-1$^{-/-}$ mice as well as LT$\alpha^{-/-}$ bone-marrow chimeras (Garcia et al. 1997; Sugawara et al. 1998; Bean et al. 1999; Kanedo et al. 1999; Saunders et al. 1999; Roach et al. 2001), correlate with rapid bacterial dissemination and reduced host survival. A characteristic feature in all of these cases is the paucity of epithelioid macrophages and failure to recruit T lymphocytes. The latter's participation in successful *Mtb* containment is underscored by a study of HIV-infected patients suffering from culture-proven tuberculous lymphadenitis (Muller and Kruger 1994). Severely lymphopaenic patients completely lacked epithelioid granulomas, while those HIV patients retaining modest peripheral CD4$^+$ counts had granulomas replete with lysozyme-expressing macrophages in apposition with CD25$^+$ (IL-2R) lymphocytes, in addition to fewer AFB. The presence of T cells not only ensures a vicinal supply of macrophage-activating T helper (Th)1 cytokines (Robinson et al. 1994; Bergeron et al. 1997; Fachetti et al. 1999; Fenhalls et al. 2000; Wangoo et al. 2001) but also raises the possibility of reciprocal costimulation. AMs in TB granulomas express high levels of the costimulatory molecules B7–1 and B7–2 with nearby T cells being CD28$^+$ (Soler et al. 1999). T cell–macrophage liaison may thus engender immunologic memory in addition to microbial killing.

2.3.2
Macrophage-Monocyte Networks: A Case for Multinucleated Giant Cells

Multinucleated giant cells (MGCs) were discovered as large, polykaryonic structures within TB granulomata over 130 years ago (Langhans 1868). Subsequently found to be a common feature in many infectious and foreign body granulomas, MGCs are thought to represent fusions between descendants of the monocyte-macrophage lineage (Anderson 2000). Indeed, MGCs can now routinely be derived in vitro using macrophages alone following the initial demonstration of rabbit AMs to fuse after treatment with supernatants from mycobacteria-sensitised lymph node cells stimulated with antigen (Galindo et al. 1974). The soluble bioactive component in this early study was most likely IFN-γ; use of recombinant IFN-γ when it became available also led human AMs to fuse which could in turn be blocked by anti-IFN-γ mAbs (Nagasawa et al. 1987).

Circulating monocytes are thought to be another source of MGCs (Gillman and Wright 1966) and they, too, appear heavily reliant on soluble factors for their coalescence (Postlethwaite et al. 1982). Again, IFN-γ seems to be the major fusogenic cytokine (Weinberg et al. 1984; Most et al. 1990; Fais et al. 1994; Gas-

ser and Most 1999; Mizuno et al. 2001) although some assistance is rendered by IL-3 (Enelow et al. 1992; Byrd 1998) and IL-4 (Takashima et al. 1993). Multinucleation also requires cell-cell contact in addition to soluble mediators, with lymphocyte function-associated antigen (LFA)-1, ICAM-1, β_2-integrins, CD44, CD47 and the macrophage fusion receptor (MFR) all posited to play a role (Most et al. 1990; Fais et al. 1994; Saginario et al. 1998; Sterling et al. 1998; Gasser and Most 1999).

That multinucleation serves some benefit against *Mtb* is suggested by the ability of IFN-γ/IL-3-treated human monocyte-derived MGCs to severely limit microbial spread (Byrd 1998). How MGCs bring about this restriction remains obscure although it could involve the purinergic $P2X_7$ receptor which is upregulated by IFN-γ (Humphreys and Dubyak 1996). Both an mAb directed against the $P2X_7$ extracellular domain and irreversible ATP analogues (^{o}ATP) block human monocyte fusion (Falzoni et al. 2000). Since stimulation of $P2X_7$ is known to promote PL maturation (see "Receptors for Activation" above), the generation of MGCs via extracellular nucleotides may be tightly coupled with an ability to dispose of the pathogen. This idea is consonant with earlier studies showing elevated lysozymal activity of MCGs within human tuberculoid granulomas (Yamashita et al. 1978; Williams and Williams 1983).

2.4 Macrophage Specialisations Within the Pulmonary Context

2.4.1 Surfactant Receptors

With a surface area approaching 50–100 m^2, the human lung mandates a very broad system of innate defence consisting of ciliary (mechanical) clearance, cough reflex (ironically serving as a vehicle for *Mtb* transmission), and cellular responsiveness. Surfactant proteins (SPs) present within the alveolar lining fluid as a means to reduce surface tension and lung collapse appear increasingly important in this non-adaptive immune repertoire (Holmskov 1999). Their relevance to TB was first noted by the ability of surfactant protein A (SP-A) to promote *Mtb* attachment and phagocytosis by AMs from both normal subjects and HIV patients (Downing et al. 1995; Gaynor et al. 1995). A second surfactant protein, SP-D, has more recently been shown to bind *Mtb* and LAM via its carbohydrate recognition domain, a feature characteristic of the collectin family to which SPs belong (Ferguson et al. 1999). SPs-A and -D have each been localised to endocytic vesicles and lysosomal granules of AMs (Walker et al. 1986; Voorhout et al. 1992), suggesting their uptake was receptor-mediated. This has proved to be the case; the human collectin receptor C1qRp and SP-R210 both bind SP-A while SP-D enters AMs via a scavenger receptor superfamily member, gp-340 (Holmskov 1999). Antibodies directed against SP-R210, in particular, inhibited SP-A-associated uptake of mycobacteria (Weikert et al. 1997) and the subsequent induction of NO and TNF-α synthesis (Weikert et al. 2000). In the pres-

ence of IFN-γ, however, SP-A treatment together with *Mtb* actually lowered RNI release (Pasula et al. 1999), while the same was seen in human AMs for peptidoglycan/TLR2-induced TNF-α secretion (Murakami et al. 2001). Thus, SPs could also have macrophage-suppressive functions which help resolve inflammation once the major bacterial burden is cleared.

2.4.2 Hypoxia-Induced Responses

Mtb is an obligate aerobe, despite being equipped with pathways for anaerobic energy metabolism (Cole et al. 1998; McKinney et al. 1998; Wayne and Sohaskey 2001). This may explain why disseminated tuberculous lesions tend to develop more rapidly in the apical lung fields of an upright host where steady-state oxygen tension is highest (Riley 1960; West 2000). Human AMs, on the other hand, remain effective phagocytes even under mildly hypoxic conditions (pO_2 >25 mmHg; ~3% O_2 at sea level) as measured in granulomas or abscess cavities remote from oxygenated blood (Cohen and Cline 1971; Hocking and Golde 1979). The environment within human macrophages may further limit O_2 availability since bacteria appear more sensitive to reduced ambient O_2 tensions when grown intracellularly (Meylan et al. 1992).

Restricted growth of *Mtb* inside macrophages could equally be interpreted from the viewpoint of the host; hypoxia elicits powerful mycobacteriostatic/cidal responses. Approximately 1% O_2 alone or in combination with IFN-γ increased NOS2 expression ~25-fold and TNF secretion 500-fold in murine and human macrophages, respectively (Scannell et al. 1993; Melillo et al. 1996). The molecular basis of these increases is in part due to hypoxia-inducible protein (HIF)-1, a transcriptional activator that functions as master regulator of mammalian O_2 homeostasis (Semenza 2001). HIF-1 binds to hypoxia response elements (HREs) in either 5′ or 3′ flanking regions of target genes following exposure to 0.5%–6% O_2 (Semenza 2001). The murine and rat NOS2 promoters, for example, both contain HREs which are bound in vitro and in vivo (Melillo 1995; Jung et al. 2000). Moreover, the earlier synergy reported for IFN-γ and hypoxia (Mellilo 1995) in promoting macrophage NOS2 expression probably stems from the recent observation that HIF-1 physically associates with IRF-1 as part of a co-activating promoter complex (Tendler et al. 2001). A cycle of stimulation is then propagated since NO enhances HIF-1α expression and DNA binding (Sandau et al. 2001), perhaps through nitrosyl–cysteine modification.

3
Harnessing the Macrophage Anti-tubercular Arsenal

3.1
Intersection of Immunity and Conventional Chemotherapy

When modern chemotherapy against *Mtb* fails, it usually does so for one of two reasons. Firstly, unsuccessful treatment outcomes may be due to acquisition of multi-drug resistance mutations on the part of the pathogen. MDR-TB accounts for only a minor fraction, however, of newly diagnosed cases: ~3% worldwide in the year 2000 (Dye et al. 2002). A second, more common problem is patient non-compliance. TB therapy calls for concomitant administration of 2–3 drugs for a minimum of 6 months. Many patients are unable or unwilling to adhere to such a lengthy drug regimen, and their neglect leads to high relapse rates since short-course therapy is insufficient to eradicate a small subpopulation of mycobacterial "persisters" (Mitchinson 1985). For this reason, the World Health Organisation has recommended that every patient receive directly observed therapy (DOT) to ensure compliance (Who 2000). Extension of the DOT strategy to individuals with latent TB infection (LTBI), however, is not practicable given the enormous number of cases involved. The problem is further aggravated by the fact that LTBI also requires a lengthy drug regimen, usually 6–12 months of isoniazid prophylaxis. To persuade a patient with active TB to complete a protracted course of antibiotics is often challenging: exhorting the same practice in an asymptomatic individual with LTBI may prove even more difficult. Clearly, development of shorter treatment regimens, especially for LTBI, is an urgent priority (Institute of Medicine 2000).

Recent studies in experimental animals suggest that post-exposure vaccination may serve as a useful adjunct to standard drug therapy (Lowrie et al. 1999; Lowrie and Silva et al. 2000). Mice infected with *Mtb* were treated with anti-tuberculosis drugs and then vaccinated with DNA encoding the mycobacterial Hsp65 heat shock protein. Such mice were completely protected from subsequent reactivation of disease, even after being treated with powerful immunosuppressive agents (Lowrie et al. 1999). Thus, in principle, immune modulation in conjunction with conventional chemotherapy could lead to a more rapid and complete cure in humans. Enhancing the efficacy of the immune response in this manner would be particularly apposite in persistent infections like TB, where natural immunity, left unmodified, does not always eliminate the pathogen (McKinney et al. 1998). The observations of Lowrie and colleagues suggest that this failure is not because the host is incapable of sterilising immunity but rather that immunity remains poorly elicited, a situation corrected with vaccination. If one could provide the requisite forms of immunostimulation, not only through vaccination but potentially via macrophage-activating agents based on cytokine signalling or downstream effectors, then new avenues of treatment for LTBI and MDR-TB could be possible in the future.

3.2
Appropriately Activating the Macrophage In Situ

3.2.1
Cytokine and Ab Immunotherapy

Parenteral administration of IFN-γ as adjunctive to antimicrobials or even as replacement therapy in immunodeficiencies such as AIDS or CGD substantiates the virtues extolled for this cytokine in what are largely experimental systems (Murray 1996). In humans, intravascular (i.v.), intramuscular (i.m.) or intradermal (i.d.) IFN-γ treatment appears well tolerated and stimulates the antimicrobial repertoire of PBMCs, circulating neutrophils and tissue macrophages. When given as an aerosol, IFN-γ activates AMs in a lung-restricted fashion (Jaffe et al. 1991). Ameliorative effects have been seen for non-viral infections caused by atypical mycobacteria (e.g. *M. avium*, *M. chelonei*) (Holland et al. 1994), and cutaneous (e.g. *Leishmania tropica*, *L. mexicana*, *L. braziliensis*) or visceral (kala-azar) leishmaniasis, in some cases yielding complete cure, especially if given with other modalities (e.g. antimonial compounds) (Murray 1996). For lepromatous leprosy, i.d. IFN-γ administration shows a clear enhancement of cell-mediated immunity, including oxidative burst, delayed-type hypersensitivity reactions and granuloma formation (Nathan et al. 1986).

In the case of TB, provision of exogenous IFN-γ could be of significant help to those patients refractory to chemotherapy. Benefits have been found for individuals with severe, advanced MDR-TB; 500 μg of aerosolised IFN-γ given thrice weekly for a month led to clear salutary effects in the form of demonstrable weight gain, reversion of positive sputum smears to negative status, and reduction in the size of cavitary lesions (Condos et al. 1997). Similar doses in other studies led to increased respiratory burst capacities and chemokine release from AMs (Jaffe et al. 1991; Halme et al. 1995), suggesting that some of the benefits in people with TB probably operate at the level of the macrophage. An improved outcome was also noted for an MDR patient receiving subcutaneous IFN-γ together with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Raad et al. 1996), as well as in TB and MDR-TB patients given inhaled IFN-α, a benefit largely thought to be due to local induction of IFN-γ (Giosue et al. 1998; Giosue et al. 2000). Whether IFNs could be administered systemically to combat extrapulmonary TB or as a reconstitutive measure in HIV-positive TB patients has yet to be examined.

Inveighing against the use of TNF as potential immunostimulant for TB is its established history as both proinflammatory and pyrogenic; indeed, these are mainly held responsible for certain clinical characteristics of the disease, namely, "phthisis" (wasting/cachexia), night sweats and tissue destruction (Keane et al. 2001). Yet its value as adjunctive agent has been suggested in some (but not all, e.g. Moreira et al. 2002) animal models: mice receiving recombinant human TNF or a non-toxic mimetic peptide (TNF_{70-80}) exhibit increased resistance to mycobacterial infection accompanied by reduced CFU, heightened NO release

and better organised granulomas (Denis 1991b; Roach et al. 1999; Briscoe et al. 2000). Small peptide mimics may also have the advantage of bypassing any sTNFR blockage (Juffermans et al. 1998) if composed of epitopes not bound by these truncated receptors. Other TNF-modulating agents (e.g. thalidomide analogues) facilitate in vivo TNF production during TB as shown by recent studies of HIV-infected patients (Bekker et al. 2000; Gotri et al. 2000).

Concern that TNF unduly promotes pulmonary tissue damage is also subject to debate; both acute and persistent TB models suggest that TNF's protective role far outweighs any pathologic involvement (Adams et al. 1995; Flynn et al. 1995; Bean et al. 1999; Mohan et al. 2001; Smith et al. 2002). In humans, too, TNF antagonists (e.g. infliximab) appear to reactivate rather than limit disease (Keane et al. 2001; Martinez et al. 2001; Wagner et al. 2002; Mayordomo et al. 2002). It could even be argued that some degree of TNFR1/2-mediated apoptosis (Mohan et al. 2001) might be useful, either through directly killing *Mtb*-infected macrophages or by liberating drug-sensitive bacteria which are more readily accessed by antimicrobials than inside the cell.

Ab therapy directed against specific *Mtb* cell wall components, e.g. LAM epitopes, is another method which could encourage macrophage activation, this time through uptake by FcγRs. An IgG3 mAb recognising arabinomannan delivered intratracheally has been shown to partially protect wild-type, IFN-$\gamma^{-/-}$ and MHC class II$^{-/-}$ mice against *Mtb* infection (Teitelbaum et al. 1998). All mAb-treated mice exhibited enhanced granuloma formation with a mantle of NOS2-positive macrophages thought to represent a barrier to bacterial dissemination. Whether mAb treatment is effective if begun after clinical signs are evident, however, remains to be determined. So, too, does the issue of whether Ab therapy might hasten immune complex deposition leading to alveolitis and other FcR-mediated lung damage (Clynes et al. 1999). In a recent study of 68 patients with active progressive TB, for example, over 50% had immune complexes in their lungs, although whether this was a primary cause or a secondary consequence of disease cannot be distinguished (Surkova et al. 1999).

As with all recombinant proteins, the utility of cytokines and mAbs is limited by the concerns of cost, bioavailability and generation of neutralising antibodies. However, at least in the case of IFN-γ, long-term administration appears feasible; CGD patients have tolerated repeated prophylactic injections for up to 7 years without noticeable decreases in plasma half-life or side-effects more debilitating than mild fever (Segal 2000). Even so, as a serious avenue for treatment of TB and activator of human macrophages in situ, cytokines and mAbs are impractical in those developing countries most affected by the disease. The real importance of the aforementioned studies is the didactic lesson they provide: enhancing macrophage anti-tubercular mechanisms can have a genuine impact on TB progression, especially in cases where chemotherapy is no longer tenable.

3.2.2
Small Molecule Chemistry: Adaptor Protein Mimics

If macrophage activation is primarily achieved through cytokine receptor signalling, why not obviate the need for a bulky ligand altogether? Small, non-peptidyl mimics could offer advantages both in terms of specificity and pharmacokinetics. Events at or downstream of IFNGRs and novel PI3 K effectors are two examples where such an approach may be applicable to the macrophage-*Mtb* problem.

Elicitation of Jaks or STATs by small molecule modulators has already been demonstrated in the case of Jak3; large-scale chemical screens were based on a known tyrosine kinase inhibitor pharmacophore and homology model derived from kinase crystal structures (Goodman et al. 1998). However, this represents but one tier of a multistage signalling cascade. In searching for modifiers of IFNGR signal transduction, for example, one could extend the screen to several levels: (1) IFNGR1/2 ligand binding and dimerisation, (2) Jak1/2 binding, (3) STAT1 homodimerisation, and (4) STAT1-DNA binding and transcriptional activation (Seidel et al. 2000). At the cell surface, small molecules capable of causing receptor dimerisation have already been found for erythropoietin and the GM-CSF receptor, in the latter case binding to alternate sites which do not compete or interfere with the natural ligand (Qureshi et al. 1999; Seidel et al. 2000).

Beyond receptor clustering lies the problem of how to confer specificity when so many components of the Jak/STAT entourage are shared among different cytokines. Here, a combinatorial approach may prove useful: drugs exhibiting higher specificity for STAT1 could be given with another of known preference for Jaks1/2. Signalling by other cytokines/growth factors would thus be minimised, since additional adaptor proteins (STATs2–5) or kinases (Tyk2, Jak3) obligate for their transduction are either poorly induced or missing. Imiquimod [1-(2-methylpropyl)-1*H*-imidazo[4,5-c]quinolin-4-amine] and its analogue S28463, antiviral drugs used against human papillomavirus and experimentally effective against vacuolar pathogens like *Leishmania* and *M. bovis* BCG, mediate their effects on macrophages preferentially through STAT1 (Bottrel et al. 1999) and MyD88 (Hemmi et al. 2002); they could serve as parent or lead compounds for more soluble derivatives. Selectivity may also be imposed at the level of transcription by targeting the STAT1 linker domain, since this region is critical for promoter complex formation following IFN-γ but not IFN-α/β stimulation (Yang et al. 1999a).

A second signalling intermediate thought to be involved in countering *Mtb* is PI_3P. This lipid directs EEA1 to the mycobacterial PG for subsequent maturation into PLs (see section entitled "Intracellular Sorting and the Conduits for Antigen Presentation and Elimination"). It also appears to bind the NADPH subunit $p40^{phox}$ for oxidase assembly on nascent PGs (Bravo et al. 2001) and could participate in IFN-γ-dependent STAT1 S727 phosphorylation (Nguyan et al. 2001). What makes PI_3P such an attractive biochemical target is its phosphoinositide headgroup interactions with protein FVYE and PX domains; they distinguish

PI_3P from all other lipids, with recognition reliant on its being embedded in a phospholipid bilayer (Misra et al. 2001). Indeed, FYVE domains exclusively bind PI_3P and no other phosphoinositide. The recent co-crystallisation of PI_3P-EEA1 (Dumas et al. 2001) and PI_3P-$p40^{phox}$ (Bravo et al. 2001) has provided structural information on which to design small molecule mimics. Novel PI_3P or p150/VPS34 (class III PI3 K) binding partners could also be isolated by selective capture on phosphoinositide affinity matrices as recently shown for PI3 kinase effectors regulating both Arf and Rho GTPases (Krugmann et al. 2002). High-throughput screens of this type may uncover compounds capable of specifically forcing macrophage PL fusion in the absence of activation.

Exploring the world of small GTPases in PL biogenesis is an avenue that promises much in the way of understanding macrophage immunity during infection with implications for drug development. GTPases are judiciously positioned to direct intracellular traffic and assist the spatial convergence of several effector pathways. For example, IFN-γ-induced Rab5a has recently been shown to remodel the PG environment of engulfed listeriae where it facilitates the translocation of Rac2 (another GTPase) to the PG surface (Prada-Delgardo et al. 2001). With Rac2 comes the NADPH oxidase and potentially NOS2 (Kuncewicz et al. 2001). Another group of IFN-elicited GTPases (the 47-kDa family) help control *Toxoplasma gondii* and *L. monocytogenes* infections in mice (Taylor et al. 2000; Collazo et al. 2001). One of these, interferon-inducible protein 1 (IFI1; LRG47), operates against *Mtb* as well (J. MacMicking, unpublished results). How IFI1 brings about its anti-tubercular effects is unknown, but mechanisms could include assisting PL fusion, Ag presentation or trafficking of lysosomal hydrolases between vesicular compartments (Fig. 2). Once determined, human IFI1 homologues would seem a logical target for pharmacologic intervention, given that its expression is limited to disease or inflammatory states making disruptions of homeostatic processes less likely. Reaching or mimicking vesicle-associated adaptor proteins like IFI1 is probably easier than attempting to deliver drugs into the *Mtb* PG lumen directly. Translocation of xenobiotics across the PG/PL membrane needs take into account substrate-specific porters and efflux pumps (Lloyd 2000); perhaps drug-conjugates utilising the transferrin receptor would enable larger molecules to gain entrance to the interior before PL maturation is complete (Clemens and Horwitz 1996).

3.2.3
Local Provision of Effector Molecules

At present no studies exist describing macrophage effector molecules being directly delivered to the pulmonary tree of TB patients. Nonetheless, one could make a case for NO or its congeners based on indirect findings. Doses as low as 70–90 ppm for 48 h kill both drug-sensitive and drug-resistant strains of *Mtb* (Long et al. 1999); this level is tolerated for up to 2 weeks in neonates with persistent hypertension, where it provides dramatic benefits (Clark et al. 2000). NO gas is preferentially delivered to the well-ventilated areas of the lung, coupling

ventilation (V) to perfusion (Q) and acting as a selective pulmonary vasodilator (Moya et al. 2001). Whether it could reach the relatively hypoxic centre of a granuloma, however, remains conjectural. Nor are the amounts of NO needed to sterilise smear-positive patients, some of whom may harbour as many as 10^5-10^6 CFU/ml of saliva or sputum (Yeager et al. 1967), known. Higher doses of NO run the risk of toxicity, especially within an O_2-rich environment where derivatives like $ONOO^-$ arise more frequently. For this reason most NO bioactivity comes in the guise of *S*-nitrosothiols (SNOs) which are relatively resistant to toxic reactions with O_2/O_2^- and are transported across the cell membrane by γ-glutamyl transpeptidases (Moya et al. 2001). It may also explain why free NO in human airway lining fluid and expired air is often below the levels required to dilate blood vessels or relax airways—the majority is complexed with glutathione and SNO-proteins which are themselves sensors of ventilatory hypoxia (Lipton et al. 2001).

An ideal drug would therefore convert NO equivalents into SNOs. It should also avoid potentially harmful peroxidation and allow the desired V/Q matching and pulmonary/systemic activity quotients (i.e. be a gas). Recently, Stamler and colleagues (Moya et al. 2001) used the following criterion to isolate such a compound: (1) high volatility, (2) resistance to oxidative decomposition, (3) low oxidising potential towards haemoglobin, and (4) biocompatibility. *O*-nitrosoethanol (ENO) was found to fulfill these criteria. It is stable at high ambient O_2 concentrations since heterolytic transfer reactions predominate over homolytic decomposition, enabling it to react preferentially with nucleophiles such as sulphurs of glutathione and proteins within the lung. It was also active under hypoxic conditions and did not affect systemic haemodynamics (Moya et al. 2001). ENO could thus represent the prototype of future compounds which allow transnitrosation reactions to occur within pulmonary granulomas in situ. The problem of delivery, however, remains uppermost on the list of limitations needing to be overcome before use against respiratory infections like TB would be considered.

3.3
Beyond Koch: *Mtb* targets as Adjunct to Macrophage Immunity

Besides attempting to enhance the macrophage armamentarium or directly furnish its products, another approach to aiding anti-tubercular immunity could include interference with the putative counter-strategies used by *Mtb* (Table 1).Traditional drugs target bacterial processes that are required for growth and division such as DNA replication or cell-wall biogenesis (McKinney 2000). This may be a critical factor limiting the efficacy of conventional chemotherapy against latent TB, where mycobacteria are thought to be in a metabolically altered and/or essentially non-replicative state (McKinney et al. 1998; Wayne and Sohaskey 2001). Agents that damage *Mtb* indirectly—by ablating RNI/ROI detoxifying enzymes, contesting Fe acquisition and storage, blocking acid tolerance or pathways involved in macrophage deactivation—may be less dependent

Table 1 Some potential *Mtb* counter-immune mechanisms as drug targets

Process	*Mtb* target[a]	Ascribed Function	Reference
1. Iron acquisition and storage	*ideR*	Fe-responsive DNA binding protein—transcriptional regulator	Gold et al. 2001
	mbtA	Siderophore—mycobactin	Gold et al. 2001
	mbtB	Siderophore—mycobactin	de Voss et al. 2000; Gold et al. 2001
	mbtI	Siderophore—mycobactin	Gold et al. 2001
	bfrA	Bacterioferritin	Gold et al. 2001
	bfrB	Ferritin	Gold et al. 2001
2. Carbon utilisation	*icl1*	Isocitrate lysase—key enzyme in the glyoxylate shunt	McKinney et al. 2000
3. Hypoxic shiftdown	*acg*	Putative nitroreductase	Purkayastha et al. 2002
	narK2	Putative nitrite extrusion protein	Sherman et al. 2001
4. Arresting PL maturation	Urease	Converts urea to NH_4 and CO_2	Gordon et al. 1980
	Others?		Reviewed in Russell 2001
5. Acid tolerance/adaptation	Urease	Converts urea to NH_4 and CO_2	Beyrat et al. 1995
	mgtC	Facilitates Mg^{2+} transport	Piddington et al. 2000
	mntH	Divalent cation transporter	Agaroff et al. 1999
	Glutamine synthetase?	NH_4 utilisation	Harth and Horwitz 1999
6. RNI detoxification	*noxR1*	Detoxifies NOx[b], GSNO	Reviewed in Nathan and Shiloh 2000
	noxR3	Detoxifies NOx[b] GSNO	Nathan and Shiloh 2000
	ahpC	Alkyl hydroperoxide subunit—detoxifies organic peroxides, NOx,[b], $ONOO^-$, GSNO	Nathan and Shiloh 2000; Springer et al. 2001
	msrA	Peptidyl methionine sulphoxide reductase	St. John et al. 2001
	lpd	Dihydrolipoamide dehydrogenase	Bryk et al. 2002
	sucB	Dihydrolipoamide succinyl-transferase	Bryk et al. 2002
7. ROI detoxification	*sodC*	Cn,Zn superoxide dismutase	Piddington et al. 2001;
	katG	Catalase-peroxidase	Edwards et al. 2001
	sigH	Alternative sigma factor, required for thioredoxin/thioredoxin reductase induction	Raman et al. 2001

Table 1 (continued)

Process	*Mtb* target[a]	Ascribed Function	Reference
	sulfatide	Cell wall component—ROI scavenger	Pabst et al. 1988
	LAM	Cell wall component—ROI scavenger	Chan et al. 1991
8. DNA repair	*nei*	Endonuclease VIII—repairs oxidative damage	Reviewed in Mizrahi and Andersen 1998
	nth	Endonuclease III—repairs oxidative damage	
	fpg	MutM formamidopyrimidine DNA glcosylase—repairs oxidative damage	
	mutY	AG adenine glycosylase—repairs oxidative damage	
	muT1	8-Oxo-dGTPase—repairs oxidative damage	
	tag1	3-methyladenine DNA glycosylase I—repairs alkylating DNA damage	
	Adl (adaA/alkA fusion)	O^6 methylguanine DNA methyltransferase (Ada domain)/ 3-methyladenine DNA glycosylase II (AlkA domain)—repairs alkylating DNA damage	
	mpg	3 methylpurine DNA glycosylase—repairs alkylating DNA damage	
	Ogt	O^6 alkylguanine DNA transferase—repairs alkylating DNA damage	
9. Avoiding Ag presentation/recognition	Unknown	Unknown	Pancholi et al. 1993; Gercken et al. 1994
	Unknown	Ag85B processing for MHC class II	Ramachandra et al. 2001
	PE-PGRS proteins	Antigenic variation	Banu et al. 2002
10. Antagonising cytokine signalling	Unknown	Disruption of STAT1 binding CBP/p300 co-activators	Ting et al. 1999
	Unknown	Inhibiting 1L-12 production	Nau et al. 2002
11. Macrophage deactivation	LAM	Induction of TGF-β, IL-10	Reviewed in Murray 1999
	LAM	Activation of SHP-1 phosphatase	Knutson et al. 1998

[a] Targets includeboth *Mtb*loci and products.

[b] NOx, unspecified nitrogen oxide intermediates.

on the growth state of the organism for their activity. A cautionary note, however, is that many bacterial species (e.g. *E. coli*, *Salmonella typhimurium*, *L. monocytogenes*) develop a general stress resistance profile upon entry into stationary phase, including mechanisms designed to deal with oxidative stress, acidic pH and other conditions synonymous with the macrophage PL environment (Nystrom 2001). Thus, it will be important to ascertain whether the same counter-strategies are operative in both dividing and non-replicating *Mtb*, a parity not necessarily observed in all bacteria (Kjelleberg 1993; Spector 1998).

Based on the aforementioned approaches, one could envisage future TB drug regimens using conventional antimicrobials, interventions that enhance or supplement endogenous immunity, and agents which further sensitise *Mtb* to killing by its human host. Such novel forms of "combination therapy" could offer several advantages over current protocols: improved effectiveness against MDR-TB, faster (shorter) treatment of active TB, and feasible strategies to address the long-neglected issue of LTBI. With respect to the last point, the successful introduction in 1997 of one-shot therapy for single-lesion paucibacillary leprosy, caused by the related pathogen, *M. leprae*, provides a relevant and inspiring precedent (WHO 1998).

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Detection and Control of Fungi by Macrophages: The Role of Carbohydrates and Antifungal Agents

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Abstract Macrophages play an important role in the innate immune response to fungal pathogens. They express receptors which recognise a variety of fungal molecular patterns, many of which are conserved cell wall carbohydrates. We present an overview of the macrophage receptors shown to be involved in fungal recognition and binding, the various antifungal mechanisms utilised by these cells, and demonstrate strategies that fungal pathogens have evolved to escape these mechanisms. We also provide an overview of the current clinical anti-fungal agents, as well as strategies which are being developed to enhance the antimicrobial mechanisms of the macrophages themselves. Finally, we discuss fungal-derived carbohydrates and their potential use as immunomodulators.

Keywords Anti-fungal agents, Beta-glucan, Carbohydrate, Fungi, Macrophage receptor

Abbreviations

H. MW	High molecular weight
CLP	Caecal ligation and puncture
↓	Decrease
↑	Increase
↔	No change

1 Introduction

Macrophages (MΦs) play a central role in the innate recognition of a range of pathogens, including fungi, and in the modulation of the subsequent effector mechanisms. Recognition generally leads to phagocytosis and killing of the invading pathogen through a variety of passive and active mechanisms. However, some fungal pathogens have learned to subvert their host's anti-microbial defence mechanisms requiring the clinical administration of anti-fungal compounds. Furthermore, with the emergence of drug-resistant strains, a greater understanding of how the immune system deals with fungal pathogens is crucial if novel anti-fungal strategies are to be developed. In this chapter, we have placed particular emphasis on the MΦ receptors involved in the recognition of fungal pathogens, as well as the killing mechanisms employed. We also discuss both current and potential strategies used to control fungal pathogens, and the role of fungal derived carbohydrates in modulating MΦ and immune responses.

Although this chapter will focus specifically on the contribution of MΦs to the innate recognition and control of fungal pathogens, these cells are not the only line of defence. Neutrophils, dendritic cells (DCs) and some lymphocytes have all been shown to play a role in the immune response to various fungal pathogens (see Vazquez-Torres and Balish 1997 for a review). Furthermore, although not discussed, the reader should bear in mind that MΦ and their specialised relatives, DCs, have a crucial role in instructing the adaptive immune response, which also plays an important role in the control of fungal pathogens (see Romani 2002 for a review).

2 Macrophage Receptors

Contact between the MΦ and fungal pathogen occurs when MΦ receptors recognise structures, such as polysaccharides, which are displayed on the fungal cell wall. The fungal cell wall, apart from giving the cell its rigidity and providing protection against environmental stress, contributes to virulence by providing a platform for adhesion to the host. The structure of the cell wall, which has been derived from the non-pathogenic fungus *Saccharomyces cerevisiae*, consists mostly of polysaccharides, including mannoproteins, β-glucans and chitin (see Fig. 1 and Chaffin et al. 1998; Lipke and Ovalle 1998 for reviews). In total, about

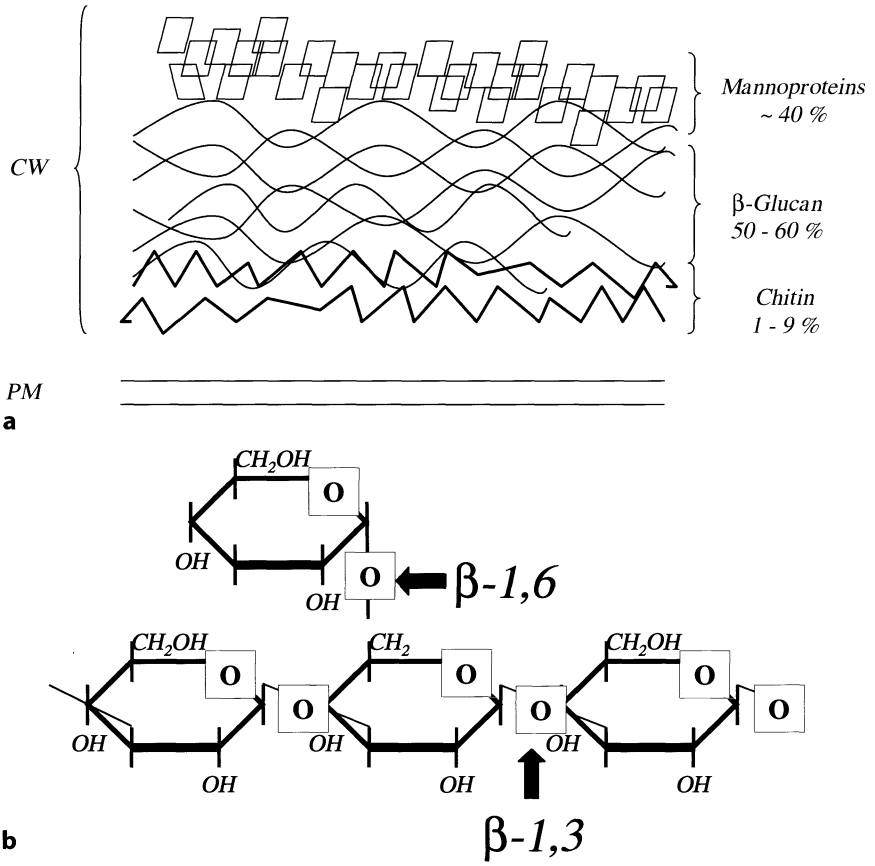

Fig. 1a, b The fungal cell wall (CW) is modular in structure and composed of various polysaccharide layers. **a** The plasma membrane (PM) is surrounded by a chitin layer (unbranched acetyl-glucosamine polymers) linked to β-glucans. Mannan is connected via di-*N*-acetylchitobiose residues to the CW protein fraction. (see Chaffin et al. 1998 and Lipke and Ovalle 1998 for reviews). **b** β-glucans form linear chains via β-1,3-linked glucose residues and branched chains via β-1,6 linkage(s)

80%–90% of the *Candida albicans* fungal cell wall is composed of carbohydrates, some of which have immunomodulatory activities, discussed in more detail below (see Tzianabos 2000 for a review).

The recognition of fungal pathogens occurs either directly via specific MΦ surface receptors or indirectly through the recognition of opsonins, such as C3 or antibodies, which coat the microbial cell surface (see Aderem and Underhill 1999; Underhill and Ozinsky 2002a for reviews). These receptors bind conserved molecular patterns that are often part of immutable structural components, such as conserved polysaccharides found within the cell wall (Medzhitov and Janeway 1997). Although complex and simple carbohydrate structures are recognised by a range of MΦ receptors (see Table 1), we will focus on those specif-

ically shown to have the ability to recognise intact fungi; the mannose receptor (MR), the β-glucan receptor (βGR), and the complement receptor (CR)3. The Toll-like receptors, which mediate intracellular signalling and resultant pro-inflammatory response, will also be discussed briefly (see Chap. 5). These receptors should not be thought of in isolation, but rather that they are simultaneously contributing to the recognition of fungal pathogens. Finally, it should be noted that the function of these receptors and their interactions remains to be fully elucidated. It is also likely that other MΦ receptors, which recognise fungal pathogens, have yet to be identified.

2.1 Mannose Receptor

The MR, often referred to as the mannose-fucose receptor, is a type I transmembrane glycoprotein, containing an extracellular cysteine-rich domain, a fibronectin type II-like domain, and eight tandem carbohydrate recognition domains (CRDs) (see Fig. 2) (see Stahl and Ezekowitz 1998 and Martinez-Pomares et al. 2001 for reviews). The MR is expressed on the majority of differentiated mononuclear cell phagocytes (see Table 1), with weak or no expression on most MΦ cell lines (Pontow et al. 1992).

The MR exhibits preferences for oligosaccharides with the following terminal residues, l-fucose>d-mannose$\geq$d-*N*-acetyl-glucosamine>>>d-galactose (Stahl et al. 1978), and displays high affinity for endogenous ligands containing branched α-linked oligo-mannoses (Kery et al. 1992; Linehan et al. 2001). MR transfectant CHO cells are capable of mediating the phagocytosis of yeast, such as *Pneumocystis carinii* and *C. albicans*, the pinocytosis of mannosylated glycoproteins (Ezekowitz et al. 1990; Ezekowitz et al. 1991) and the binding of *Cryptococcus neoformans* mannoproteins (Mansour et al. 2002). The MR also exists as a soluble cleaved form capable of binding the *S. cerevisiae*-derived particle, zymosan, as well as *C. albicans* (Martinez-Pomares et al. 1998). While both endogenous and exogenous ligands have been identified for the MR (see Table 1), its full role in the binding of yeast remains unresolved (see Linehan et al. 2000 and Martinez-Pomares et al. 2001 for reviews). Experimental conditions under which fungal binding was assayed often did not take into account the presence of other mannose-binding lectins, such as Nkcl/Dectin-2 (Fernandes et al. 1999; Ariizumi et al. 2000a).

The MR is believed to signal via its 45-amino acid cytoplasmic tail, via as yet unknown mechanisms (Ezekowitz et al. 1990). The pro-inflammatory cytokines interleukin (IL)-6, granulocyte-MΦ colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α and IL-12 can all be generated after phagocytosis of fungi (Stein and Gordon 1991; Garner et al. 1994; Shibata et al. 1997; Yamamoto et al. 1997). The MR is not, however, responsible for the production of certain chemokines, such as MΦ inflammatory protein (MIP)-1β, MIP-2 and KC (a platelet factor 4 neutrophil chemoattractant family member) during *C. albicans* infection, indicating that other receptors are involved in fungal-mediated

Table 1 Macrophage lectinswith known and potential fungal derived carbohydrate binding ability (modifiedfrom Linehan et al. 2000)

Receptor	Expression	Ligands	Carbohydrate	Reference
β-glucan receptor (Dectin-1)	Myeloid cells	*S. cerevisiae, C. albicans*, unidentified ligand on T cells,	β-1,6-Glucans and β-1,3-glucans	Ariizumi et al. 2000b; Brown and Gordon 2001; Willment et al. 2001
Mannose receptor (mannosyl-fucosyl receptor)	Differentiated MΦs, lymphatic and sinusoidal endothelium, cultured DCs, perivascular microglia, and mesangial cells	Sialoadhesin, lysosomal proteases, glycosidases, peroxidases, yeasts and fungi	Fucose, mannose, N-GlcAc, galactose	Ezekowitz and Stahl 1988; Linehan et al. 2001; Linehan et al. 1999; Martinez-Pomares et al. 2001; Stahl 1992
MΦ galactose receptor	Peritoneal and tumoricidal MΦs	Tumour Tn antigen microbes	Galactose or N-GlcAc terminal oligosaccharides	Suzuki et al. 1996
CR3 (Mac-1, CD11b/CD18)	Lymphocytes, MΦs, NK cells, neutrophils	Microbes, ICAM-1, fibrinogen	Non-specific sugar binding	Muto et al. 1993; Ross 2000; Ross et al. 1985a; Ross and Vetvicka 1993; Thornton et al. 1996
Nkcl (Dectin-2)	MΦs, monocytes, neutrophils, langerhans cells	Unknown	Mannose-sepharose	Ariizumi et al. 2000a; Fernandes et al. 1999
Mouse MΦ C-type lectin	Primary MΦs and cell lines	Unknown	Unknown	Balch et al. 1998
Galectin-3 (Mac-2)	MΦs	IgE and other host molecules, *C. albicans*	β-1,2-Linked oligomannosides	Cherayil et al. 1989; Fradin et al. 2000

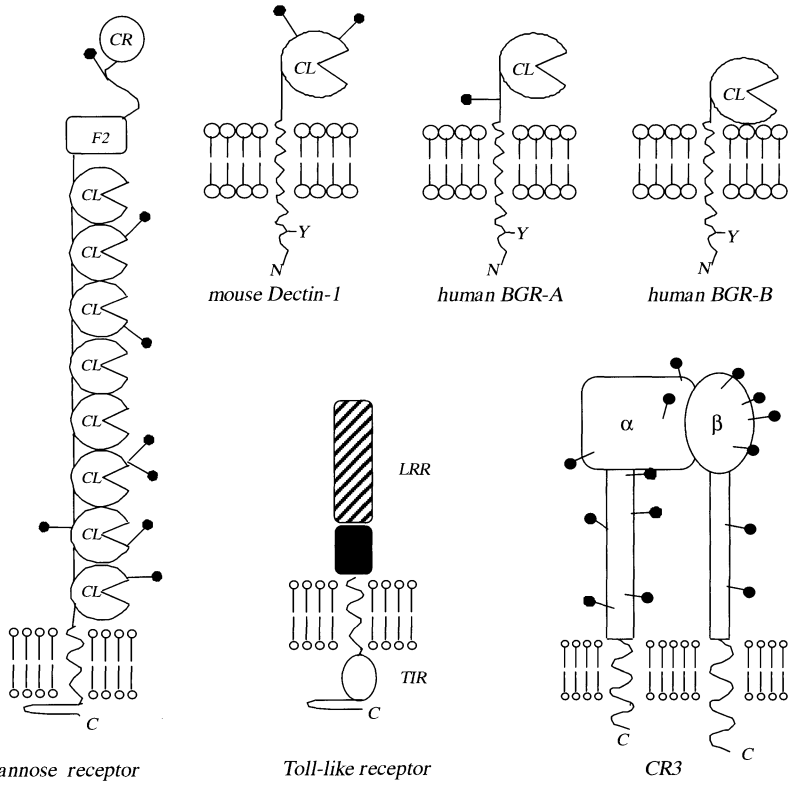

Fig. 2 Schematic representation of the MΦ receptors involved in fungal recognition. The following abbreviations and symbols represent protein domains, their various modifications, and important motifs: *CL*, lectin C-type; *α*, CD11b integrin subunit; *β*, CD18 integrin subunit; ●-, N-glycosylation sites; *F2*, fibronectin type II-like repeats; *Y*, ITAM motif; *LLR*, leucine-rich repeat C-terminal domain; *CR*, cysteine-rich domain; *TIR*, Toll-interleukin 1-resistance intracellular signalling domain. The protein orientation relative to the lipid bilayer (*open lollipops*) is indicated by the *N* (N-terminal) and *C* (C-terminal) ends

signalling (Yamamoto et al. 1997). These data support the concept that MΦ receptors act in concert to generate the appropriate signalling response.

2.2 The β-Glucan Receptor

The existence of a non-opsonic zymosan and yeast-binding β-glucan inhibitable monocyte/MΦ receptor was first demonstrated two decades ago (Czop and Austen 1985a,c; Czop and Kay 1991). Recently, a trypsin sensitive C-type lectin receptor, Dectin-1, was demonstrated to be a MΦ βGR (Brown and Gordon 2001; Brown et al. 2002; Willment et al. 2001). Dectin-1 is a type II transmembrane protein with a single CRD, and a cytoplasmic tail containing an immunomodulatory tyrosine-activating motif (ITAM) (see Fig. 2) (Ariizumi et al. 2000b). Mu-

rine Dectin-1 is expressed in a number of cell types; including DCs, monocytes, MΦs, neutrophils and even a small sub-population of T cells (Brown and Gordon 2001; Taylor et al. 2002).

Dectin-1 recognises a variety of carbohydrates containing β-1,3 and/or β-1,6 linked glucans, as well as an unidentified endogenous ligand, so far described only on T cells (Ariizumi et al. 2000b; Brown and Gordon 2001; Willment et al. 2001). Murine NIH3T3 fibroblasts transduced with the human and murine form of Dectin-1 were shown to recognise and internalise intact *C. albicans* and *S. cerevisiae* blastospores, in a β-glucan dependant fashion (Brown and Gordon 2001; Willment et al. 2001). As most fungi contain β-glucans within their cell wall (see Douglas 2001 for a review), it is likely that they interact with Dectin-1. Indeed, a number of pathogens have been reported to interact with β-glucan binding receptors, including *P. carinii*, *C. neoformans* and *Aspergillus fumigatus* (Kan and Bennett 1991; Cross and Bancroft 1995; Vassallo et al. 2000).

The interaction of fungi with the βGR results in the activation of MΦs and the release of pro-inflammatory mediators and cytokines (see Czop 1986 and Williams 1997 for reviews). These stimulatory effects appear to be linked to the structure of the β-glucans (see below) and may be mediated by the ITAM motif in the cytoplasmic tail of this receptor (Brown and Gordon 2001). Furthermore, the β-glucan content of fungi varies depending on their morphological state which results in the production of different cytokine and chemokine profiles (for example see Torosantucci et al. 2000). A more comprehensive list of the effects of β-glucans on the immune system is presented in Table 2.

2.3 Complement Receptor 3

CR3 (Mac-1) is a heterodimer composed of two chains, CD11b, which is unique to CR3, and CD18, which is common to all β2 integrins (see Fig. 1). CR3 is widely expressed in monocytes, MΦ, DCs, neutrophils, eosinophils, natural killer (NK) cells, some $CD8^+$ T cells and $CD5^+$ B cells. It has diverse functions (see Table 1) ranging from mediating migration of myeloid leukocytes and NK cells, to the phagocytosis and killing of complement opsonised microbes (see Ross and Vetvicka 1993; Ross et al. 1999, 2000 for reviews). Unlike the β-glucan or mannose receptors, CR-mediated recognition does not directly result in the release of pro-inflammatory responses, such as the production of reactive oxygen intermediates and arachidonic acid, implying that other receptors are involved in mediating these responses (Wright and Silverstein 1983; Aderem et al. 1985; Aderem and Underhill 1999).

Most fungi become opsonised with C3b by activating the alternative and mannose-binding protein pathways, or though the classical (antibody-mediated) pathway (see Kozel 1996, 1998 for reviews). As CR3 has a binding site for the C3b component of complement, it plays an important role in the recognition of opsonised fungi. In addition, CR3 possesses a lectin domain on CD11b, which binds a broad range of carbohydrates as well as unopsonised yeast particles

Table 2 Immunomodulatory effectsof β-glucans

Model	β-Glucan treatment	Structure	Post-treatment	Response	Reference
J774a.1	Glucan-phosphate	Soluble	LPS	$\downarrow$ NF-κB	Williams et al. 2000
RAW 264.7	Grifolan	H. MW	None	$\uparrow$ IL-6, $\uparrow$ IL-1 and $\uparrow$ TNF-α	Adachi et al. 1994
RAW 264.7	Grifolan	Insoluble	None	$\uparrow$ TNF-α	Ishibashi et al. 2001
Murine resident peritoneal	β-Glucan	Particulate	None	Superoxide release, $\uparrow$ IL-1α	Gallin et al. 1992
MΦ	β-Glucan	Soluble	Particulate glucan	No effect	
Mice	β-Glucan	Soluble	LPS, staphylococcal enterotoxin B, toxic shock syndrome toxin1	$\downarrow$ TNF-α, $\downarrow$ IL-6	Soltys and Quinn 1999
Mice	Curdlan sulphate	Soluble	LPS	$\downarrow$ TNF-α, $\downarrow$ IL-1β, $\leftrightarrow$ IL-10, $\leftrightarrow$ IL-6	Masihi et al. 1997
Mice	Grifolan	H. MW	LPS	$\uparrow\uparrow$ TNF-α	Ohno et al. 1995
	SSG	H. MW			
	OL-2	H. MW			
Mice	β-Glucan	Soluble	CLP	$\downarrow$ NF-κB, $\downarrow$ IL-6	Williams et al. 1999
Rats	PGG-glucan	Soluble	*S. aureus*	$\leftrightarrow$ TNF-α, $\leftrightarrow$ IL-1β, $\uparrow$ clearance of infection	Liang et al. 1998
U937	Zymosan	Particulate	None	$\uparrow$ NF-κB	Kadish et al. 1986
Human monocytes	Yeast particles	Particulate	None	$\uparrow$ TNF-α, $\uparrow$ IL-1β	Abel and Czop 1992
Human monocytes	Zymosan	Particulate	None	$\uparrow$ leukotrienes	Janusz et al. 1987
	Yeast-derived	Soluble	Particulate glucans	Decrease	
Human mononuclear cells	β-Glucan	Particulate	None	$\uparrow$ IL-1R agonist, $\leftrightarrow$ IL-1β	Poutsiaka et al. 1993
	β-Glucan	Soluble	Particulate	Decrease IL-1R agonist	
Human dermal fibroblasts	Glucan-phosphate	Soluble	None	$\uparrow$ NF-κB, $\uparrow$ IL-6	Kougias et al. 2001
Rabbit alveolar MΦ	Zymosan	Particulate	None	$\uparrow$ arachidonic acid	Daum and Rohrbach 1992
	β-Glucan	Soluble	Particulate glucan	Decrease	
Rabbit alveolar MΦ	β-Glucan	Particulate	None	$\uparrow$ TNF-α	Olson et al. 1996

(Ross et al. 1985a; Thornton et al. 1996). The importance of complement and CR3 in the recognition and control of fungal infections is highlighted by genetic defects which result in decreased resistance to a variety of fungal pathogens, including *C. neoformans*, *C. albicans*, *A. fumigatus* and *Paracoccidioides brasiliensis* (Ross et al. 1985b; Kozel 1996, 1998). However, as uptake via CR3 also bypasses the cellular defence mechanisms mentioned above, this receptor is also targeted by a number of fungal pathogens, including *H. capsulatum*, *C. albicans*, and *Blastomyces dermatitidis*, as a means of invading the cell (Hogan et al. 1996).

2.4 Toll-Like Receptors

Innate immune specificity is thought to be determined by homo- or heterodimers of Toll-like receptors (TLR) (see Fig. 2), which cooperate with phagocytic receptors, such as the MR, to trigger a pathogen-specific response (Ozinsky et al. 2000; Takeuchi and Akira 2001; Underhill and Ozinsky 2002b; Vasselon and Detmers 2002). Toll receptors were originally identified as key components of the antifungal response in Drosophila (Lemaitre et al. 1996). Although a number of TLRs have now been identified in mammals (Medzhitov 2001), only a few have so far been implicated in fungal recognition (Underhill et al. 1999). TLR-2 was shown to participate in the immune response to zymosan as well as gram-positive bacteria, peptidoglycans and lipoarabinomannan. However, heterodimers of TLR-2, and TLR-6 are required for the complete activation of the transcription factor nuclear factor-kappa B (NF-κB), and the subsequent TNF-α production during the detection of zymosan (Underhill et al. 1999; Ozinsky et al. 2000).

TLR-4 was originally thought to be involved in the recognition of bacterial components, such as lipopolysaccharide (LPS) and lipoteichoic acid (Underhill and Ozinsky 2002b), but has recently been shown to be involved in the recognition of fungal pathogens as well (Shoham et al. 2001). The requirements for TLR-4-induced signalling, however, vary depending on the pathogen and cell type examined (Wang et al. 2001; Netea et al. 2002). In *Candida*-treated peripheral blood mononuclear cells and murine MΦs, TLR-2, but not TLR-4, is involved in the production of the pro-inflammatory cytokines, TNF-α and IL-1β (Netea et al. 2002). However, TLR-4-defective mice are more susceptible to *C. albicans* infection, perhaps as a result of the reduction in the production of various chemokines required for neutrophil recruitment in these mice (Netea et al. 2002). Similarly *C. neoformans* glucuronoxylomannan activates NF-κB via TLR-4 and CD14, as examined in transfected fibroblast cells, but the activation does not result in TNF-α production (Shoham et al. 2001). In contrast, *A. fumigatus* hyphae induce TNF-α, IL-1β and IL-6 production in human leukocytes via TLR-4 and CD14, but not via TLR-2 mediated pathways (Wang et al. 2001). Overall, these data suggest that a wide range of fungi, and consequently a diverse range

of potential ligands, can be recognised by a variety of Toll-like receptors, a process which leads to the appropriate immune response.

3 MΦ Killing and Fungal Avoidance Mechanisms

The recognition and binding of fungal pathogens, via the various MΦ surface receptors, leads to phagocytosis and the implementation of a variety of anti-microbial mechanisms. Killing is achieved by a combination of oxygen-independent mechanisms, such as the low pH, degradative environment and limitation of nutrients within the phagosome, as well as oxygen-dependent mechanisms, including the production of reactive oxygen and nitrogen intermediates.

The production of reactive oxygen intermediates is an effective antimicrobial mechanism against fungi, such as *C. albicans* (Hampton et al. 1998; Clark 1999). Superoxide (O_2^-) is generated by the activated membrane-associated enzyme complex phagocyte NADPH oxidase (phox), which transfers electrons from NADPH to O_2. Toxic oxidants, such as hydroxyl radicals and hydrogen peroxidase (H_2O_2), are then generated within the phagosome killing the internalised organism. Mutations of the phox proteins, resulting in chronic granulomatous disease, are defined by the lack of in vitro phagocyte killing and the recurrence of bacterial and fungal infections, highlighting the importance of this antimicrobial mechanism (Leijh et al. 1977; Borgato et al. 2001). The role of superoxide acting as a signal for the activation of neutrophil granule proteases, and not as a toxic intermediate, was recently shown to be essential for bacterial killing and may also prove to be important in fungal killing (Reeves et al. 2002).

Myeloperoxidase (MPO) catalyses the production of hypochlorous acid, a potent microbicidal agent, from H_2O_2 (see Hoy et al. 2002 for a review). MPO is located within the granules of monocytes and neutrophils and is involved in their fungicidal and anti-microbial functions (Marodi et al. 1991; Hampton et al. 1998). It may function as a regulator of the oxidative degradation process, by the sequestration of H_2O_2 in the phagosome, thereby facilitating granule protease activity (Reeves et al. 2002). Although MΦs do not synthesise MPO, recombinant human MPO has been shown to enhance killing of *Candida* by GM-CSF-activated MΦs (Marodi et al. 1998). There may also be a link between the ability of the MΦ MR to bind MPO and its role in antifungal mechanisms (Shepherd and Hoidal 1990).

The inducible nitric oxide (NO) synthase (iNOS) plays a key role in the killing of pathogens (see MacMicking et al. 1997 for a review). iNOS enables the production of NO via the oxidative deamination of l-arginine. The NO then reacts with superoxide, or with thiol groups to produce peroxynitrite and nitrosothiols. A combination of NO, superoxide and peroxynitrite seems to be required for the full MΦ fungicidal activity (Vazquez-Torres and Balish 1997). Induction of iNOS occurs in the presence of microbial products, such as LPS and zymosan, or pro-inflammatory cytokines, such as TNF-α, IFN-γ and IL-1β. The importance of iNOS in controlling infections is evident by the use of specific

NOS inhibitors, which generally exacerbate infections (MacMicking et al. 1997). Furthermore, NOS knock-out mouse models display an increased susceptibility to fungal infections, such as *C. neoformans* (Rivera et al. 2002).

In addition to these oxygen-dependent killing mechanisms, the non-oxygen-dependent mechanisms also play an important role in the control of fungal infections. The confinement of microbes within the phagosome acts as a physical barrier limiting the availability of nutrients, whose supply is further restricted by active mechanisms. For example, the transferrin receptor, which transports iron via the endosomal pathway, is down-regulated during infection, while the recruitment of the natural resistance-associated MΦ protein (Nramp)-1 to the phagosome ensures the removal of iron and other essential divalent cations (Gruenheid et al. 1997; Blackwell and Searle 1999; Sunder-Plassmann et al. 1999). The degradative enzymes and acidic pH of the phagosome contribute to fungal killing, although these mechanisms have yet to be fully characterised (Vazquez-Torres and Balish 1997). The iron content and pH of the phagosome have also been targeted for anti-fungal therapy, discussed below.

Although MΦs are very efficient at killing microbial invaders, fungal pathogens have subverted many of these killing mechanisms to ensure their survival. Some fungi actively seek intracellular residence in a modified phagosome, such as *H. capsulatum*, which survives and replicates intracellularly by regulating the phagosomal pH (Newman 1999). Other fungi ensure that they avoid recognition, such as *C. neoformans* which has a viscous polysaccharide capsule predominantly composed of glucuronoxylomannan that protects against recognition by phagocytes (Perfect et al. 1998). Other avoidance mechanisms include the ability to suppress iNOS, as occurs in *C. albicans* infections (Vazquez-Torres and Balish 1997; Schroppel et al. 2001), and the production of compounds that interfere with phagocytosis, such as the aflatoxins and gliotoxins of *A. fumigatus* (Tomee and Kauffman 2000).

4 Antifungal Agents

While only few fungi are considered "professional pathogens", many are opportunistic pathogens capable of infecting immunocompromised individuals, such as AIDS patients (van Burik and Magee 2001). To combat these infections, two general antifungal strategies have been pursued, those that target the fungus itself and those that enhance the microbicidal activity of phagocytes. Often the simultaneous use of these strategies results in improved efficacy (for example see Tanida et al. 2001). Most drugs in clinical use directly debilitate the fungus and include those that target fungal cell membrane sterols, such as the polyenes (including amphotericin B), azoles (including fluconazole), allylamines and morpholines. Drugs which target fungal cell-wall synthesis include compounds such as the echinocandins and pneumocandins, which target β-glucan synthesis, and nikkomycin Z, which inhibits chitin synthesis. Other antifungal drugs include 5-fluorouracil, which interferes with RNA, DNA and protein synthesis.

For a more complete review on these and other antifungal agents see Ghannoum and Rice (1999).

Although not in routine clinical use, a number of studies have indicated that modulation of phagocyte function by immunotherapy can be beneficial for the management of systemic fungal infections. A number of cytokines, including GM-CSF, G-CSF and IFN-γ, have been shown to enhance phagocyte antifungal activity (Marodi et al. 1993; Farmaki and Roilides 2001; Roilides and Farmaki 2001). Furthermore, pretreatment with cytokines, such as GM-CSF, could reduce the risk of disseminated fungal infections in patients therapeutically immunosuppressed with drugs such as corticosteroids (Brummer et al. 2002). Other potential antifungal drugs include compounds which modify the phagolysosome, such as chloroquine, which by altering the phagolysosomal pH and iron availability, is effective against fungal pathogens such as *Histoplasma capsulatum* (Weber et al. 2000). Finally, as mentioned above, carbohydrates derived from the cell wall of the fungi themselves also show promise as immunomodulators for the control of fungal infection (Williams et al. 1978; Williams et al. 1991; Garner and Hudson 1996).

5 Immunomodulation by Fungal-Derived Carbohydrates

There is a considerable body of research documenting the effects of fungal-derived carbohydrates on the immune system, and an interest in generating novel therapeutics based on these compounds. These effects were initially noted over 40 years ago with zymosan, which is rich in α-mannan and β-glucans, and now is widely used as a particulate activator of MΦs (Benacerraf et al. 1959; Czop et al. 1989). More recent studies have shown that mannoproteins have immunomodulatory properties (see below), whilst soluble β-glucans can have mitogenic, anti-infective, anti-sepsis, and anti-tumourigenic effects (Riggi and Di Luzio 1961; Williams and Di Luzio 1980; Browder et al. 1987; Sherwood et al. 1987; Sandula et al. 1995; Williams et al. 1996; Kogan et al. 1997; Ross et al. 1999).

Fungal mannans have both suppressive and stimulatory activities on the immune system (Garner et al. 1990; Delfino et al. 1996; Delfino et al. 1997). Mannan is often associated with protein, which may be contributing to the immunomodulatory effects (Palma et al. 1992; Gomez et al. 1996; Tzianabos 2000; Mansour et al. 2002). As a stimulator, mannans can interact with phagocyte receptors, such as the mannose receptor, inducing the production of pro-inflammatory cytokines, such as TNF-α and IL-6 (Garner and Hudson 1996; Tzianabos 2000). The suppressive activities of mannans have been documented in patients suffering from *Candida* or *Cryptococcus* infections (Tzianabos 2000). Although the suppressive mechanism is not clearly understood, it is thought to involve interactions with T lymphocytes (Garner et al. 1990; Tzianabos 2000).

β-Glucans have potent stimulatory effects on the immune system, although the degree of immunomodulation is linked to structure of these carbohydrates

(Bohn and BeMiller 1995; Williams et al. 1996; Kogan et al. 1997; Williams 1997). The tertiary structure, degree of branching, polymer length and carbohydrate content have all been implicated in the ability of a particular β-glucan to stimulate the immune system. These stimulatory effects are probably related to the ability of the glucan to cross-link the βGRs on the phagocyte surface (Okazaki et al. 1995; Mueller et al. 2000). Unfortunately, the detailed structure of most immunomodulatory β-glucans and the molecular mechanisms by which they exert their effects are unknown, hampering efforts to develop these compounds for therapeutic use. In general, the administration of β-glucans results in phagocyte activation and the production of pro-inflammatory mediators, such as TNF-α (Czop and Austen 1985b,c; Browder et al. 1990). A list of some of these glucans and their immunomodulatory effects is presented in Table 2.

6 Conclusions

MΦs have an important role in the innate immune response to fungal pathogens, and consequently they express a number of receptors, which are able to recognise fungal molecular patterns. The identification of these receptors has greatly aided the understanding of the mechanisms by which the immune system recognises these pathogens and how the ensuing cellular and immune response is generated. These studies have also provided insight into the mechanisms by which fungal pathogens subvert the immune system and have led to the development of therapeutics, including the use of fungal-derived carbohydrates as immunomodulators.

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