

Yoshiro Maru

Inflammation and Metastasis



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This book is dedicated to my wife

Preface

In this book, I attempt to describe a way for readers to contemplate how to approach the complicated issue of inflammation and tumor progression.

As of January 30, 2015, the precision medicine initiative by President Obama was manifested [1]. My understanding is that one of the objectives would be elucidation of complex mechanisms underlying human diseases by integrating environmental factors. Understanding cancer metastasis by inflammation that is caused by both environmental and endogenous elements is one such issue described in this book. A more precise classification of disease into subgroups as discussed by advisory board members may correspond to distinction between prometastatic and nonmetastatic subgroup of a given tumor and patient [2].

Summarizing what is written in the literature without including his own ideas often results in an ambiguous table of lists showing the names of molecules based on publication without interpretations. I was an active professor of more than 10 years before official retirement and still struggle to cope with experimental difficulties. Originally, I am a physician specialized in hematology and started my research on oncogenes and human leukemia in 1985. I obtained my thesis on the discovery of Eph tyrosine kinase in 1987. After spending a postdoctoral career at UCLA under Dr. Owen N. Witte from 1989 through 1993 working on the BCR-ABL oncogene of chronic myeloid leukemia (CML), I began my research on angiogenesis at the University of Tokyo with Dr. Masabumi Shibuya. By particularly focusing on VEGF receptor 1, I also learned about extracellular matrix and its interacting molecules, such as proteases. Since I became a chair of the Department of Pharmacology at Tokyo Women's Medical University in 2002, I can afford to continue my research on CML, Eph, and VEGF. This has made my research background of leukocyte, tumor biology, and angiogenesis. Since 2004, I have been focused on tumor metastasis in collaboration with researchers of innate immunology. Thus, I would like to underline that I challenge the big issue of metastasis by still working on and combining the experimental experience of tumor biology, angiogenesis, and immune system from both clinical and basic standpoints. This book was not designed to collect all of the information on inflammation, tumor, immune system, and infection. It is just impossible, and I

apologize to those whose excellent work I was unable to cite simply because of space.

Some of the milestone reviews of excellence are as follows. The tumor biology started with discoveries of oncogenes and tumor-suppressor genes in the research background of tumor development in animals and cell transformation, particularly from the standpoint of cell proliferation [3]. In the course of the progress, pleiotropic activities of tumor suppressors were nicely described by Sherr, including not only mitogenic signaling but also cell-cycle checkpoint, DNA repair, and protein stability [4]. In 2011, Hanahan and Weinberg described eight hallmarks of cancer, i.e., sustained growth, evasion of growth suppressors, resisting cell death, replicative immortality, tumor angiogenesis, metastasis, genetic instability, inflammation, metabolic reprogramming, and immune alteration [5]. Among those, Massague focused on the most life-threatening feature: metastasis [6]. He and his colleagues described how infiltrating cancer cells as seed need to cope with deadly soil as metastatic microenvironment. One such way is to acquire what they called metastatic traits, such as mutations and alteration of expression levels of responsible genes in metastatic tumor cells.

Currently, we cannot understand cancer without knowledge of inflammation (Part III and Part IV). Inflammation and cancer is just like the chicken and egg. In 2001, inflammatory responses to cancer were introduced and blockade of inflammatory cytokines and chemokines was proposed for cancer treatments by Balkwill and Mantovani [7]. They described in *Cancer Cell* in 2005 that polarized macrophages and persistent inflammation underlie in cancer [8] and in *Nature* in 2008 how oncogenes induce inflammation by referring to expression of cytokines-chemokines and their receptors [9]. Also in 2002, Coussens described wound healing as an example of what they called physiological inflammation [10]. Cancer-associated inflammation was proposed to give an opportunity for therapy. The concept of physiological inflammation is closely linked to what Matzinger proposed in 2002 as danger hypothesis [11]. The idea that the immune system is concerned with danger but not non-self was proposed by Matzinger back in 1994. Although she described dangerous self and harmless foreign, tumors are recognized as neither associated with microbial stimulators nor able to send alarm signals. Endogenous alarmins, i.e., tumor-derived transformed and therefore dangerous selfs were not stated (Part II).

In 2008, Medzhitov reported endogenous inducers of inflammation, such as uric acid, S100A8, and HMGB1 [12]. An idea called para-inflammation also was proposed in which leukocytes help a tissue to adapt to stress for its restoration. Takeuchi and Akira focused on pattern recognition receptors for microbe-derived exogenous ligands, in which they did not include receptors, such as PTX3, CRP, and CD36 [13]. An idea of endogenous ligands or danger-associated molecular pattern (DAMP) was described by Karin [14]. The paper reported the bidirectional signaling between immune and tumor cells.

Inflammation has both pro- and antitumorigenic effects, the balance of which may determine the fate. Protumorigenic activities often are under vicious cycle with

NF κ B, which is involved from tumor development to metastasis. They discussed DAMP derived from surgery, chemotherapy, and irradiation against tumors. For therapeutic standpoint, Dinarello summarized anti-inflammatory agents but without any particular focus on given diseases [15].

The idea of premetastasis cannot be explained by danger hypothesis simply because premetastatic microenvironment is a condition without tissue destruction and before arrival of true danger as tumor cells and may include still reversible periods of para-inflammation (Part IV). The whole spectrum, including physiological inflammation, which is homeostatic and reversible, para-inflammation and danger-induced inflammation is proposed as homeostatic inflammation [16, 17].

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Part I

What Is Inflammation

Chapter 1

General Thinking About Inflammation

1.1 What Is Inflammation?

Currently, the definition of “inflammation” is ambiguous. In ancient medicine, a tumor may be distinguished between inflammatory or neoplastic from the pathological standpoint. When you look at recent scientific literatures, even if the title contains the word “inflammation,” the actual story of the paper is the gene expression analysis of a group of chemokines. This means that “chemokine” is just representing a concept: “inflammation.” Originally, chemokines are growth factors that can stimulate leukocyte mobilization. We currently understand that most of the vascular permeability factors have the chemokine feature at the same time. I believe and therefore propose that it can be safely said that leukocyte mobilization and/or vasopermeability is necessary and sufficient for inflammation. Chapter 1 focuses on this issue.

You may know a common disease called acute tonsillitis. Tonsil is a piece of defense machinery in the body against extrinsic assaults, such as microbes. The patients have infectious bacteria, including *Haemophilus influenzae*, a well-known Gram-negative rod, which causes sore throat, lymph node swelling in the neck, and frequently fever up to 40 °C. We now face at least three issues. One, *Haemophilus influenzae* is commensural bacteria, in other words, a resident in the pharynx of healthy individuals. This means that microbes that can potentially cause disease may coexist with the host. Why does it happen? Second, although the primary focus of the disease is tonsil in the oral cavity, why does the temperature measured by inserting a thermometer into axilla or rectum change from the normal temperature of 36.5 °C to 39 °C? Fever is generated not only in the tonsil but also in the whole body. Third, why do the lymph nodes distant from the tonsil become larger with pain?

To address those fundamental questions of daily occurrence, we need to have an accurate understanding of what takes place in the local tissues of infection and how the host responds to the microbial invasion. We need to have so much information and logics to learn from the standpoint of various hallmarks of inflammation.

In addition, I need to refer to one more important aspect of the acute tonsillitis described. The word <acute>, which is usually utilized by confronting with <chronic>, indicates that inflammation as a phenomenon is not only a space- but also time-dependent process. The duration of an acute or chronic disease depends on the disease. Acute tonsillitis and viral infection, such as influenza usually ameliorates within a few weeks. Chronic influenza never exists, but chronic viral hepatitis persists for more than 6 months until the patient dies unless treated properly by modern medicine. The essential point is that acute inflammatory states can be restored to the state before. However, if the tissue destruction caused by the inflammation is severe, as found in grave herpes zoster, the restoration is partial and sometimes leaves abnormal circumstances, such as allodynia, in which pain-sensing neuronal fibers unfortunately anastomose with touch-sensing ones during repair after destruction causing pain on touch. Chronic inflammation is based on the persistent presence of the causes of the inflammation.

Collectively, the following three essential elements should always be in mind to understand the inflammation-associated diseases that will be described in this book: cause, space, and time. The purpose of this book is not to describe every single inflammatory disease but to extract fundamental elements from them and extend the idea in the background of tumor biology by proposing a new idea of metastasis.

1.2 Essence Suggested by the Origin of the Word “Inflammation”

What is the essential meaning of the word <inflammation>? We go back to the article <Treatment for the symptoms caused by fever> in *De Medicina* written by Aulus Cornelius Celsus (ca25BC – ca50), a scientist in ancient Roma, approximately 2000 years ago [1]. We will see the well-known description of four features of inflammation: rubor (redness), tumor (swelling), calore (heat), and dolore (pain). He also stated that fever does not always accompany inflammation. In modern medicine, it is believed that the center for body thermoregulation is localized in the anterior hypothalamus, and therefore, the temperature is affected even if measured in the axilla distant from the tonsil. In the infected tonsil, those four features can be observed.

Long before the description by Celsus, the concept of inflammation already existed in Greek before the Roman conquest. Although the actual levels of contribution of Hippocrates (ca 460BC – ca 370BC) are not clear, the word inflammation appears in Hippocratic Collection. Considering that the concept that tumor is caused by abnormal cell growth has been recognized in the recent 200 years after the dark scientific era in Europe, inflammation is a more basic phenomenon pertinent to humans, whereas tumor is an emerging disease. Thus, it is easily understood that inflammation is now extensively discussed in tumor biology,

especially in the field of metastasis, which is the most difficult part to conquer and awaits mechanistic elucidation.

<Go back to Hippocrates> is frequently referred to the clinic because of the importance of his ethical view underlying the practical medicine and human dignity since the eighteenth century. In addition, I can see in the principle proposed by Hippocrates an analogous one that can be utilized to make a better understanding of what we have learned from the numerous results of molecular biological analysis. Hippocrates expressed inflammation as movement of fluid [2]. φλεγμονη means inflammation in modern Greek, and φλεγμα means body fluid. In the <epidemics> section of Hippocrates Collection, ρωδεξ (discharge) from the eyes is translated as inflammation by the translator W.H.S. Jones. Hippocrates correlated an occurrence of inflammation in wound with levels of excretion of body fluid. Harmful fluid was believed to be in the blood and proper passage of pus from the wound could accelerate the cure. Hippocrates tried to explain the mechanism of inflammation by the imbalance of four elements in the body, i.e., fluid, blood, bile, and water. His interpretation is not based on abnormality of single body fluid but on the observation of the phenomenon as a whole, a functional combination of multiple elements. For example, referring to a modern disease, chronic myeloid leukemia (CML), is not equal to the BCR-ABL oncogene (see Chap. 9). BCR-ABL, which is the most extensively studied human oncogene, against which the most leading molecular-targeted drug has been developed, causes imbalance of cytokines responsible for the normal differentiation and growth of blood cells and genome instability leading to further generation of genetic alterations. What we face as clinical manifestations in CML is a conglomeration of disturbed homeostasis in cytokine-associated cell cycle and apoptosis, DNA, and so forth. Although I suppose that nobody thought about leakage of substance from vessels in the time of Hippocrates, the leakage of fluid from the space A to B as a phenomenon simply indicates microenvironmental changes of both A and B eventually losing their balance.

I change the four elements in the theory by Hippocrates to four modern elements in molecular biology: leukocytes, vessels, coagulation, and local resident cells. The weight of contribution for each in inflammation changes in a manner dependent on time and space as a view in a kaleidoscope. Now, I will explain how the four features of Celsus correspond to what I propose as four elements of inflammation.

1.3 Pathological View

You cannot understand the basis of inflammation without taking a look at the local tissues, if any, in which the inflammation actually takes place. Histopathological findings change depending on the cause, the spatial position within the inflammation, and the time passed after the onset.

For example, microbial invasion induces gaps approximately 100–300 nm in length in the endothelium as an initial change, which results in the leakage of intravascular contents with water into extravascular space. The major substance

that passes through the endothelial barrier is albumin. The albumin is of ellipsoid structure with radius around 3×8 nm. The leaked fluid is called <transudate> whose protein concentration is low. The vascular permeability is enhanced by a variety of molecules (see Chap. 3), and I believe that this is one of the essential hallmarks of inflammation. As the inflammation proceeds, larger proteins are allowed transendothelial movement into extravascular space, including almost all the serum proteins, such as coagulation factors, represented by fibrinogen whose shape is long ellipsoid of $10\text{--}40$ nm $\times$ 3 nm. At this time point, the leaked fluid is called <exudate> with increased protein concentration (Fig. 1.1). Those transendothelial movements of intravascular substances, including proteins and water, are histologically detected as amorphous intercellular media and clinically recognized as edema. The exudate contains fibrin that is generated by the coagulation cascade (see Chap. 3), white blood cells, and occasionally red blood cells.

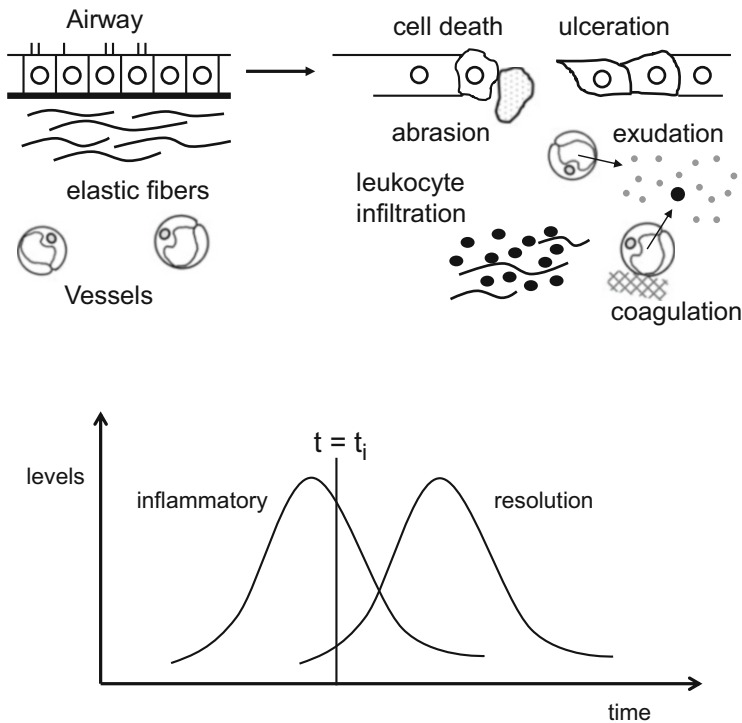


Fig. 1.1 Airway inflammation. The space underneath the epithelial cell monolayer of respiratory tracts has basement membrane, extramatrix fibers, and vessels in normal conditions. Upon infection of microbes, such as influenza virus, ulceration and cell death of the layer results in barrier destruction and increased vascular permeability induces exudates, clot formation, and leukocyte infiltration, mainly lymphocytes in influenza, in the subepithelial space. This inflammatory phase is followed by the resolution phase that puts an end to and regenerates the damaged tissue. The time (t) in the image is $t = t_i$, i.e., around the peak of inflammatory phase at which resolution is already in execution

The sizes of white and red blood cells are 10–20 μm and 7.5 μm , respectively. As we will see in Part 3 where I will convince you that tumor tissue induces persistent inflammation, a mean size of the endothelial gaps within a tumor is reported to be approximately 1.7 μm . It is of note that the sizes of blood cells are by far larger than the size of endothelial gaps. In fact, there are destruction of vessels in the inflammation sites allowing passive migration of blood cells as represented by bleeding. However, even in the absence of the destruction active migration processes exist, especially for white blood cells.

Damage of the vascular barrier, which is mainly formed by endothelial cells, leads to extravasation of serum proteins and blood cells. To stop this harmful circumstance, there exist host defense mechanisms of thrombus-mediated patchwork for the damaged and therefore leaky sites by platelets and coagulation factors. In the meantime, the damaged vasculature is fixed, thrombus is lysed, and the repair is completed. Fibrin is an end product of the coagulation cascade, and by working in concert with platelet aggregation makes a major contribution to the formation of thrombus.

Mobilization of white blood cells from bone marrow to the site of inflammation takes place within a few hours after the onset. Now, we have another essential hallmark of inflammation: transendothelial cell migration. Enlargement of vascular lumen results in an increase of blood flow and facilitates the vascular permeability. This explains the redness, heat, and swelling at local inflammatory sites. An increased tissue pressure as well as induction of pain-inducing substances, such as bradykinin, stimulates their receptors on the C fiber responsible for nociception.

Let's return to the fundamentals that I referred to at the beginning of this chapter. When we think of the essential elements of inflammation that I proposed (cause, space, and time), the four features of Celsus (redness, heat, swelling, and pain) are sufficient for inflammation but not prerequisites. In the aforementioned acute tonsillitis caused by *Haemophilus influenzae*, the site of infection lacks changes caused by recruited white blood cells at the very early time point before their mobilization. Reversely, in chronic inflammation as represented by pulmonary tuberculosis caused by *Microbacterium tuberculosis*, levels of redness and heat due to vasodilatation and swelling due to exudates are very low, and patients seldom suffer from inflammation-induced pain. Chronic inflammation sites usually carry macrophages instead of neutrophils.

Those pieces of information raise fundamental questions about [1] the essential differences between acute and chronic inflammations, and [2] the dynamic property of acute inflammation. The first question cannot be understood without the answer to the second question. Wound healing process, which is usually self-limited and restoration is possible within a certain period of time, can provide information on acute inflammation.

1.4 Dynamic Features of Acute Inflammation

1.4.1 Wound Healing

The wound healing process consists at least three phases: inflammatory phase in a restricted sense, tissue generation phase, and tissue remodeling phase. In the inflammatory phase, vessel destruction activates coagulation system, including platelet activation and leukocyte infiltration, to battle against unexpected microbial invaders. This needs to be ended to avoid too much cellular responses. The counteraction is executed in the resolution phase, which is concurrent with or followed by the tissue generation and remodeling phases (Fig. 1.1). For instance, overactivation of leukocytes may induce too much reactive oxygen species (ROS) to further damage the originally injured tissue. In the tissue generation phase, granulation develops by endothelial cells, macrophages, and fibroblasts followed by reepithelialization. In the tissue remodeling phase, those kinds of cells are rearranged in a functional manner to synthesize extracellular matrix (ECM) to regenerate the tissue. This cycle needs to be eventually closed. Any imbalance in the cycle may trigger a different round of the cycle, and cycling in a longer period of time may display chronic inflammation.

1.4.2 Simple Models of Wound Healing in Primitive Lives

We can learn lessons of inflammation from primitive species, such as sea urchin eggs and *Caenorhabditis elegans*, during injury with or without microbial invasion and bleeding. In syringe punctate experiments with sterile needles in sea urchin eggs, vacuoles that are generated in a Ca^{2+} -dependent manner in the cytoplasm migrate to and seal the holes originated by the damages [3]. The basic mechanism that underlies the phenomenon is a membrane fusion strategy to form the barrier between intra- and extracellular spaces.

In *C. elegans*, which is always exposed to hydrostatic pressure, a generation of holes is lethal. To defend against this danger, elevation of intracellular concentrations of Ca^{2+} in the damaged and its surrounding areas plays an essential role in sealing the holes [4]. Different from sea urchin eggs, epithelial cell layers form the barrier between host and environment in *C. elegans*. It is of note that Ca^{2+} appears to be the initiator of molecular events in sealing the holes in both primitive species. The increment of Ca^{2+} eventually activates actin cytoskeleton, which is mediated by the small GTP-binding proteins, such as CDC42 and Rho. The subsequent morphological changes and acquisition of migration potential in epithelial cells are the molecular basis of the sealing.

It is clear that both organisms utilize hole-sealing strategies in a manner dependent on Ca^{2+} and cytoskeletal molecules. Although those primitive modes of repair for injury lack the complicated array of inflammation observed in mammalian cells,

it safely can be said that both sensing and fixing the structural damages in cells and tissues are a principal process that is conserved through evolution. This needs to be remembered when we think of inflammation in mammalian cells.

In place of immune cells, epithelial cells have the capacity to fix the damage. The process lacks the essential features of inflammation, such as long-distance cell migration and active movement of fluid. Therefore, it is likely that the activities in the inflammatory phase after sensing the injury in wound healing may be required for the following two phases in mammals but can be skipped or dispensable in primitive organisms lacking immune cells.

1.4.3 Wound Healing Models in Mammals

Needless to say, the most common inflammation in substerile condition, at least in principle, is what is caused after surgical operation. In clinically big operations, the bleeding volume is closely associated with the postoperational prognosis of the patient. Bleeding that is accompanied by surgical operation is due to manual destruction of vessels, which is followed by inflammation.

The immediate early response of neutrophils in inflammation involves cell-to-cell interaction, because they need to be attached to endothelial cells at certain sites and then penetrate through the endothelial barrier. This process is regulated by the cell adhesion molecule (CAM) family of proteins, including cadherin, selectin, integrin, and immunoglobulin (Ig) CAM superfamily of proteins (Fig. 1.2).

A good lesson is learned from patients of leukocyte-adhesion deficiency type I syndrome. Those patients suffer from recurrent bacterial and fungal infections and impaired wound healing caused by the severe defect of leukocyte recruitment to the sites of infection and tissue damage [5]. The molecular basis of this genetic disorder of recessive inheritance is a variety of gene mutations in CD18, which is the common $\beta 2$ subunit of integrin to CD11a/CD18, CD11b/CD18, CD11c/CD18, and CD11d/CD18. Those integrins are expressed in both neutrophils and monocytes. For example, the counter-receptor of CD11b/CD18 (Mac-1) is intercellular adhesion molecule-1 (ICAM-1) expressed in endothelial cells (Fig. 1.3).

In CD18-KO mice, excisional wounds that were punched by biopsy stamps failed to recruit neutrophils that combat invading bacteria. Although monocyte emigration is largely normal, the monocyte-derived macrophages have a reduced number of apoptotic neutrophils to eat and therefore are less activated to secrete TGF β that is required for cell differentiation of myofibroblasts essential for granulation tissue contraction [6]. This TGF β -mediated process includes the tissue formation and remodeling phases in wound healing followed by the inflammatory phase. TGF β has both positive and negative effects in inflammation in wound healing. For example, wound closure and granulation tissue formation were impaired in TGF β -inducible early gene 1 (TIEG1)-KO mice [7].

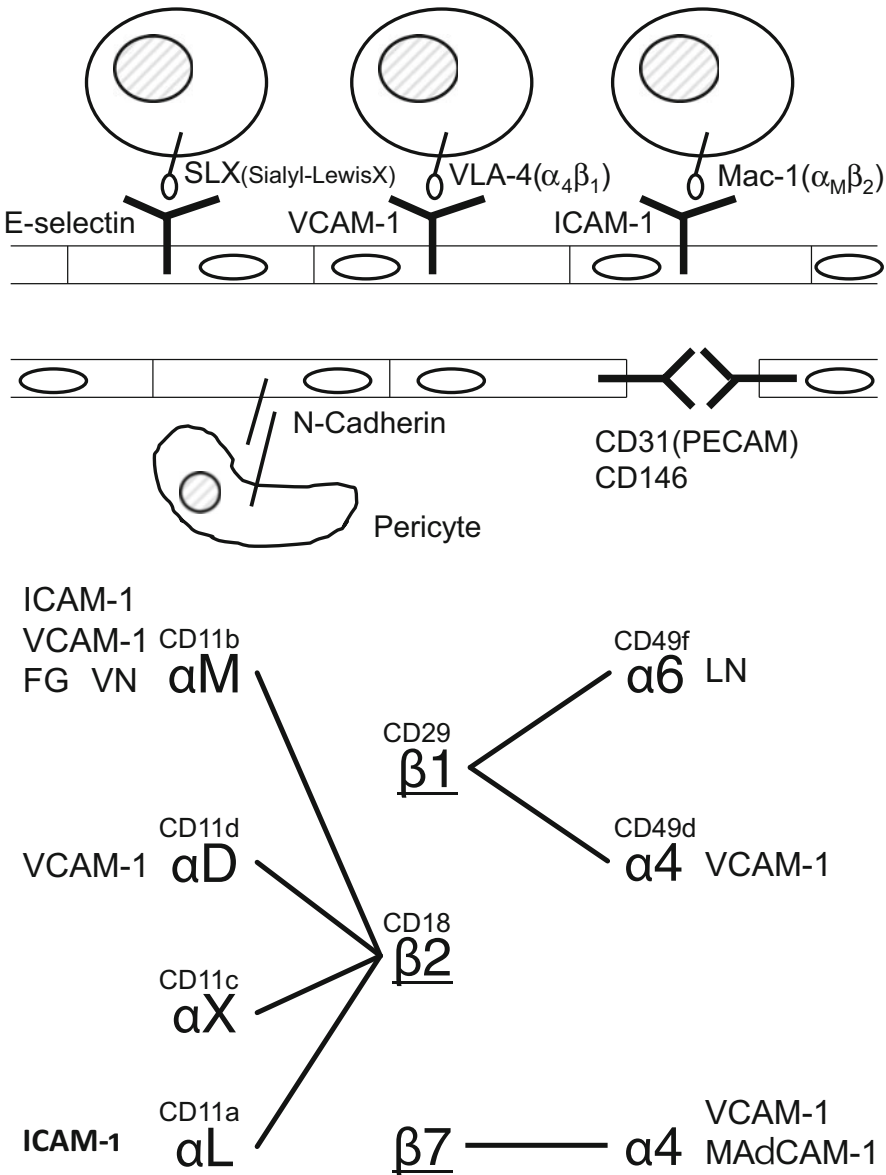


Fig. 1.2 Adhesion molecules. Endothelial cells (EC) express E- and P-selectins that bind counter-receptors expressed in leukocytes E-selectin ligand-1 (ESL-1) and P-selectin glycoprotein ligand-1 (PSGL-1), respectively. L-selectin (also called CD62L) binds counter-receptors, such as glycoprotein GlyCAM-1 and CD34 expressed endothelial cells, and is cleaved by ADAM17. VLA-4 (very late antigen-4) is $\alpha_4\beta_1$ integrins. Mac-1 is $\alpha_M\beta_2$ integrins, also called CD11b-CD18, and binds VCAM-1. N-cadherin mediates interaction between EC and pericyte. Inter-EC adhesion is mediated by CD31 (also called PECAM). Generally, selectins recognize sialylated sulfated O-linked oligosaccharides called sialylLewisX oligosaccharides (SLX), which is generated via a series of biochemical cascade, lastly catalyzed by α [1, 3] fucosyl-transferase Fuc-TVII. Lymphocyte function-associated antigen-1 (LFA-1) is CD11a-CD18 and binds ICAM-1

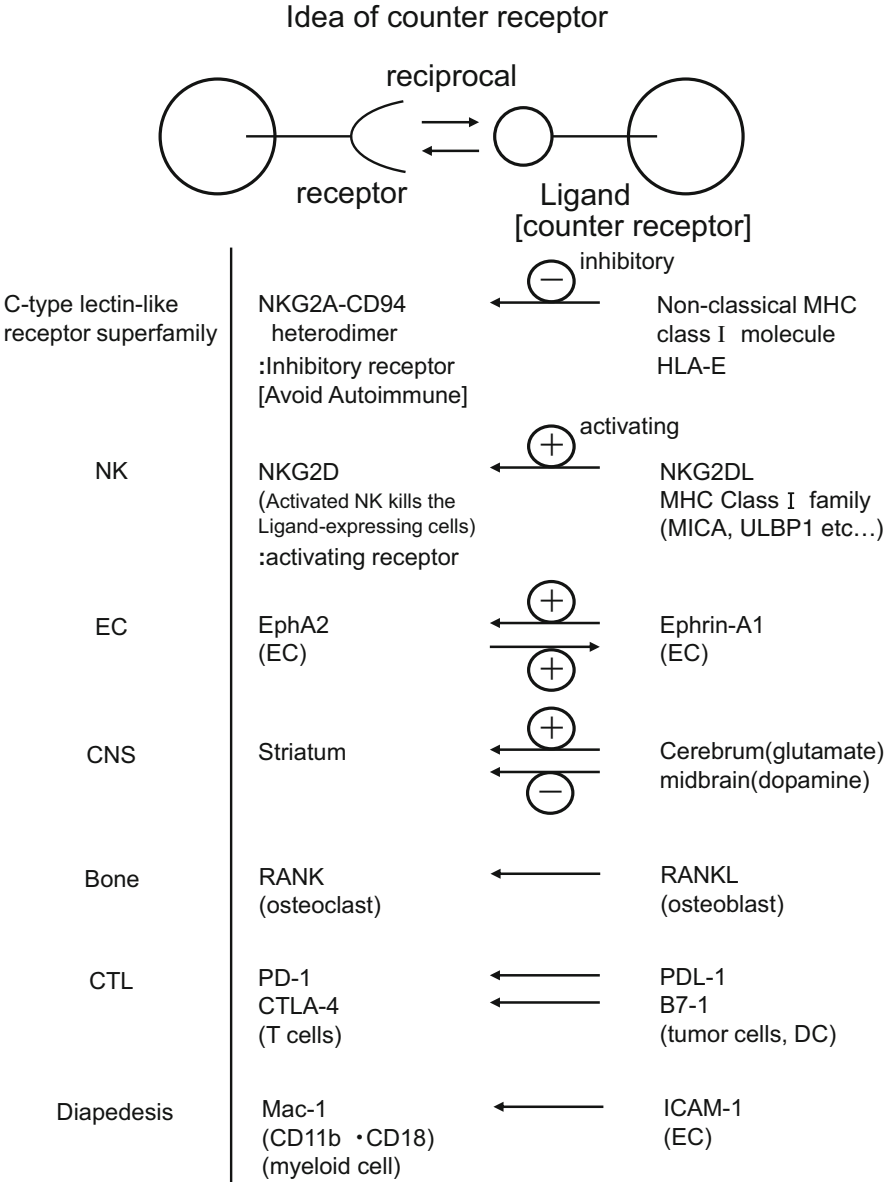


Fig. 1.3 Idea of counter-receptor. Both transmembrane proteins expressed in separate cells bind each other to transmit signaling possibly in both directions. The pair of VCAM-1 and its counter-receptor $\alpha 4 \beta 1$ integrin, shown in Fig. 1.1, and CD40 and CD40 ligand (CD40L, also called CD154) (see Fig. 8.3 in Chap. 8 for the TNF family) also are examples in this system. NK cell receptors are a good example to understand this system, because the biological outcome is clear, i.e., killer activity against target cells. Low-level and ubiquitous expression of MHC class I, HLA-E in human, is recognized by the NKG2A-CD94 complex in NK cells, which results in negative effects on the NK cells to avoid autoimmune activation. In contrast to the inhibitory receptor, NKG2D, which is not coupled with CD94, recognizes overexpressed NKG2D ligands, such as MICA and ULBP1, by oncogenes, such as HER2 and H-RasV12, and is activated.

1.4.4 *Lipid Mediators*

The discovery of lipid mediators that promote resolution or catabasis of the inflammatory disease has given a clear picture of the existence of the active process to end the precedent inflammatory phase. Prostaglandin E2 (PGE2), which is synthesized from arachidonic acid by Cyclooxygenase (COX) in the inflammatory phase, induces vascular permeability and leukocyte mobilization, directly and/or indirectly via mast cells. A homeostatic negative feedback against this event is caused by PGE2-stimulated up-regulation of 15-LOX, which is responsible for production of resolution-promoting or pro-resolving lipid mediator lipoxin (LX) A4 in charge of counteracting against leukocyte migration [8]. Other resolution mediators in charge include resolvin (Rv) (Fig. 1.4), chemerin, and annexin A1 [9].

In a murine model of full-thickness excisional wound, elevated chemerin expression was induced in epidermis, which peaked at day 4. It exerts chemotactic activity via CMKLR1 (chemokine-like receptor 1). Importantly, chemerin is subjected to proteolytic cleavage by serine proteases, such as neutrophil elastase to generate anti-inflammatory and pro-resolving peptides, including chemerin 15 (C15). C15 promotes phagocytosis of microbes through its receptor ChemR23 in macrophages and neutrophils.

In the wound model, ChemR23 expression also was up-regulated in granulation tissue, which was sustained for 14 days [10]. Topical administration of C15 not only diminished adhesion of both neutrophils and platelets as assessed by fluorescence spinning-disk intravital microscopy but also accelerated wound closure by changing the pattern of fibroblast-produced collagen deposition from an aligned bundle observed in scar to basketweave appearance found in unwounded skin. C15 reduces scarring. Macrophage phenotypes were shifted to M2 (see Chap. 2), which is engaged in efferocytosis, i.e., dead cell disposal. RvE1 is known to stimulate ChemR23 as a receptor. ChemR23-KO mice displayed increased recruitment of neutrophils but no change in wound closure. In addition, another peptide derived from chemerin carboxyl-terminus was found to have a structural homology with antimicrobial cathelicidin and direct a bacteriolytic effect.

Search for second messengers of glucocorticoid (GC) to ameliorate inflammation uncovered <lipocortin> that was induced and released, which was later renamed as annexin with Ca²⁺-binding domain. Annexin-A1-KO mice have elevated COX-2 expression, and neutrophil migration was dramatically enhanced when peritonitis was induced by zymosan [11]. In a mouse carrageen-induced

Fig. 1.3 (continued) Although it is not counter-receptor, a similar dichotomy is found in the central nervous system (CNS), and it constitutes a universal mode of cell interaction. Activation status of striatum is balanced by glutamate-mediated positive signaling from cerebrum and dopamine-mediated negative signaling from midbrain. In Parkinson disease that accompanies degeneration of neurons in substantia nigra in the midbrain, the positive signaling balances over the negative, giving pathological phenotypes

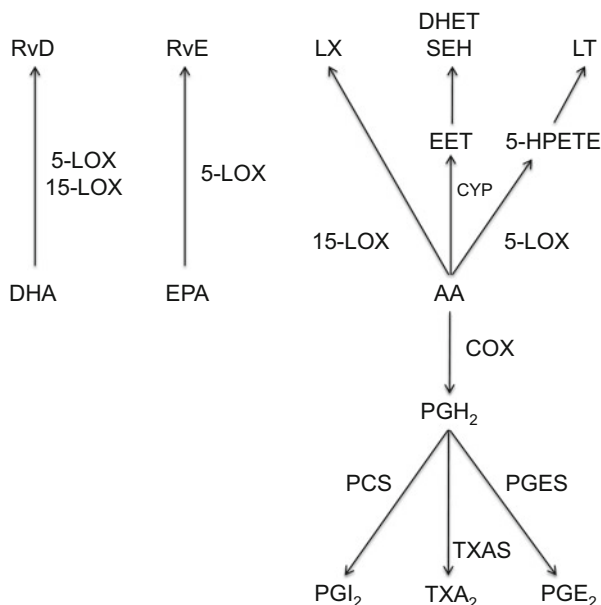


Fig. 1.4 Eicosanoid metabolites. Eicosa and tetra means 20 and 4 in Greek, respectively, and the trivial name arachidonic acid (AA) is an eicosatetraenoic acid (ETA) in the systemic name indicating 20-carbon fatty acids with four double bonds. Eicosapentaenoic acid (EPA) also is called timnodonic acid with five double bonds. Docosahexaenoic acid (DHA) is likewise 22-carbon fatty acids with six double bonds. Through enzymes, such as lipoxygenases (LOX), prostacyclin synthetase (PCS), PGE synthetase (PGES), and AA is metabolized to a variety of active lipid mediators, such as leukotrienes (LT) including LTA4, LTB4 and Cys-LT (See details in the text)

edema model in paw, the first phase of edema, which is neutrophil-mediated, peaked at 5 h, can be inhibited by GC. The GC effect was abolished in Annexin-A1-KO mice. Delayed wound closure was observed in Annexin-A1-KO mice. Formyl peptide receptor 2 (FPR2) is a receptor for Annexin-A1, LXA4, and RvD1 (see Note in Chap. 16).

1.5 How to Think of Chronic Inflammation

The cause of an inflammatory event determines the rest of the features of the inflammation, i.e., space and time. If the inflammatory response of the host fails to eliminate the cause completely for any reason, for example, by the poor ability of the host due to genetic mutations or by immunological evasion by the cause as represented by hepatitis C virus and human immunodeficiency virus, the inflammation persists and is called chronic. In addition to the proinflammatory causes, we need to consider lack of pro-resolving programs to explain and ultimately strategize against the inflammation that supports cancer. Tumors were described as <wounds

that do not heal> by Harold F. Dvorak in 1986, which may be explained by this idea.

What would happen when resolution is altered? Keloid, a tumor-looking scar unique to humans, may provide insight. Enriched in mesenchymal cells, collagen bundles, infiltrating T cells, and cytokines, such as TGFβ1 and IL-6, keloid seldom regresses and frequently recurs even after resection. Although it does not metastasize to other organs, keloid-derived precursor cells (KPC) from patients, which can regenerate connective tissues after transplantation, appear to exist as in the case of cancer-initiating cells (see Chap. 9) [12]. KPC express IL-6 in an autocrine manner to induce Oct-4 and hTERT (see Chap. 10 for telomere). IL-17 produced by Th17 cells (see Chap. 2) activates IL-17 receptor on KPC to regulate the IL-6 loop. Cooperation between IL-6 and TGFβ induces Th17 differentiation.

1.6 Markers of Inflammation

The markers include erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum amyloid A (SAA), and white blood cell count (Table 1.1). They may partially reflect causes of inflammation, but they are more useful to monitor the clinical course of the disease.

Increased ESR mainly reflects anemia, elevated γ-globulin, and fibrinogen. Electrophoretic tests of serum protein tell us that fibrinogen is included in the β-globulin fraction and immunoglobulin and CRP in the γ-globulin fraction. Fibrinogen is a well-known acute phase reactant as well as the final molecule in the coagulation cascade. CRP, one of the most reliable markers of inflammation,

Table 1.1 Inflammation markers

	CRP	SAP	PTX3	SAA1/2
t _{1/2}	19 h	24 h	<10 h	1.25–1.5 h
Serum level	0.8 μg/ml	30~50 μg/ml	2 ng/ml	1~5 μg/ml
	↓ 500 μg/ml		↓ 200–800 ng/ml	↓ 1000 μg/ml
Expression	Liver	Liver	DC, EC, MΦ, Epi	Liver, Epi
LPS	–	+	–	+
Fibronectin	+	+	–	?
C1q	+	+	+	
Influenza	-	+	+	+

Basic information of some inflammatory markers in clinical and subclinical settings is shown. The most useful CRP has a plasma half-life of 19 h and a drastic up-regulation in human but not mice. CRP binds fibronectin and C1q but not LPS. In contrast to liver production in CRP and SAP, PTX3 is generated everywhere in the body by various types of cells including dendritic cells (DC), endothelial cell (EC) and macrophages (MF) and epithelial cells (Epi) [13]. Interestingly, PTX3 is stored in neutrophils for ready-to-use. PTX3 belongs to the pentraxin family protein consisting of short pentraxin such as CRP and SAP, and long one as PTX3. SAP also binds influenza virus

belongs to the short pentraxin family of protein that forms a homopentamer [13]. Activated macrophage-derived cytokines, such as IL-1 and IL-6, stimulate hepatocytes to produce CRP and SAA to levels more than 1000-fold over the normal state in human. It is of note that rodents are poor responders of CRP. CRP also is produced extrahepatically in vascular smooth muscle cells and adipocytes. Presence of inflammation also is indicated by activation of the complement cascade, the major roles of which are to mobilize leukocytes toward the exogenous microbes and dead cells and to stop expansion of the inflammation by their disposal. This is to maintain the stability of the affected tissues. Both SAA1 and SAA2 produced in liver are contained in HDL and engaged in cholesterol (C) metabolism [14]. Acyl CoA: cholesterol acyltransferase (ACAT) participates in the esterification of C to C ester (C-E) and its storage. SAA not only inhibits this process but also activates neutral cholesterol ester hydrolase (nCEH), which converts C-E to C facilitating the extracellular transport of C via ABCA1. Old textbooks state that the human SAA3 gene is a pseudogene and that SAA3 proteins do not exist in humans, but recently we have shown its physical and functional existence in a special form (see Chap. 15 for details). Murine SAA3 is produced extrahepatically and integrated in HDL, although in a smaller amount than SAA1 and SAA2.

Chapters 2 and 3 focus on leukocytes, vessels, and coagulation and provide their fundamental information before we plunge into the chapter about tumor and its relevance to inflammation.

1.7 Concluding Remarks

The four pathological features proposed by Celsus—redness, swelling, heat, and pain—are based on increased vascular permeability and leukocyte recruitment. Currently, we need to understand inflammation as interplay among vessels, leukocytes, coagulation system, and tissue resident cells from the standpoint of cause, space, and time. Wound healing models are useful for inflammation experiments. Basic information on adhesion molecules of leukocytes and eicosanoids is provided.

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Chapter 2

Inflammation from the Standpoint of Leukocytes

2.1 Definitions

Leukocytes – Leukocytes are white blood cells of both myeloid and lymphoid origin. Polymorphonuclear (PMN) cells are differentiated myeloid cells consisting of neutrophils that are predominant, eosinophils, and basophils.

Microbes – The term microbe is used to refer collectively to any [microscopic organisms](#), including [bacteria](#), [fungi](#), and [viruses](#), unless otherwise indicated specifically as bacteria or virus.

2.2 Introduction

Herein, I state on the basic knowledge of immunology with influenza as an example. Immunity is essentially a defense and repair program of host against extrinsic invaders. We are constantly exposed to microbial and chemical assaults from the environment. Microbial invasion could be too disastrous for the targeted tissue to be restored back to the condition before or even lethal to the life. Severe bacterial pneumonia could cause life-threatening respiratory failure. Whether lethal or not, the immune response takes place but the host may die before it is accomplished.

Then what is the relationship between inflammation and immunity? I suppose that inflammation includes a part of the immunological event. Levels of tissue damage and immunological response determine those of inflammation. In microbial invasion, levels of leukocytes and the antibody against it in the peripheral blood have been of clinical value. Antibody production by lymphocytes also is one of the leukocyte activities. Differentiation of leukocytes from hematopoietic stem cell (HSC) is shown in Fig. 2.1. In addition to morphology and cell surface markers, information to delineate functionally leukocyte subgroups has made the scheme

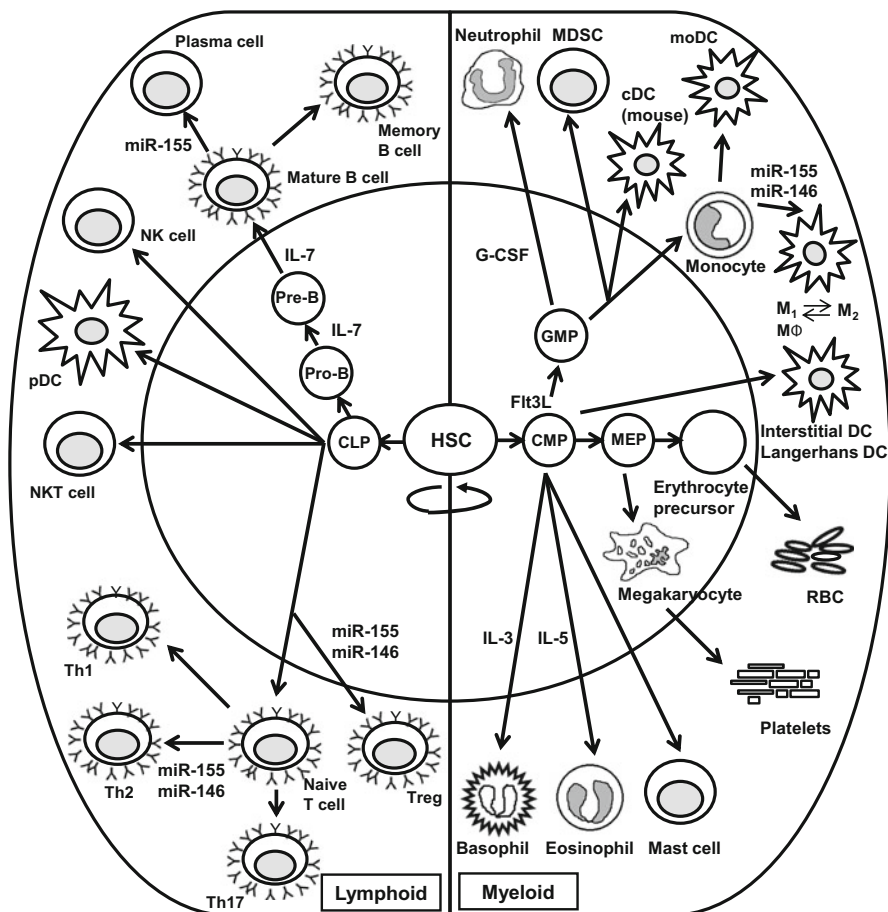


Fig. 2.1 Hematopoietic cell differentiation scheme. Differentiation of dendritic cell (DC) is complicated and still needs complete determination. Mainly mouse DCs were depicted. They are classified as conventional DC (cDC) and plasmacytoid DC (pDC). cDC is further subgrouped as (1) $CD8\alpha^+ CD103^+$ cDC that develop in a Flt3L-dependent manner, whose expressions are mutually exclusive being $CD8\alpha^+$ DC in lymphoid tissues and $CD108^+$ DC in nonlymphoid ones, and (2) $CD11b^+$ cDC enriched in lymphoid organs. Nonconventional DCs include (1) monocyte-derived DC (moDC), also called inflammatory DC (iDC), which is $MHCII^+ CD11b^+ CD11c^+$ as well as monocytic markers $CD64^+ Fc\gamma RI^+$, and pDC, which was initially identified in humans (see text) and thought to be derived from CLP but certainly share cDC properties. pDCs express CD11c and BST2. Mast cells migrate to skin and mucosa to get mature and are involved in anaphylaxis. Some cytokines and microRNA (miR) that are involved in the differentiation are shown. Whereas osteoclasts originate from HSC, osteoblasts are of mesenchymal origin. GMP granulocyte-monocyte progenitor; CMP common myeloid progenitor

rather complicated. Leukocytes grow in bone marrow, but the sites of action are inflammatory tissues with vessels as their route in between. Vessels are described in the next chapter. Laboratory tests in clinical settings assume what is happening in the inflammatory site of interest by the indirect information.

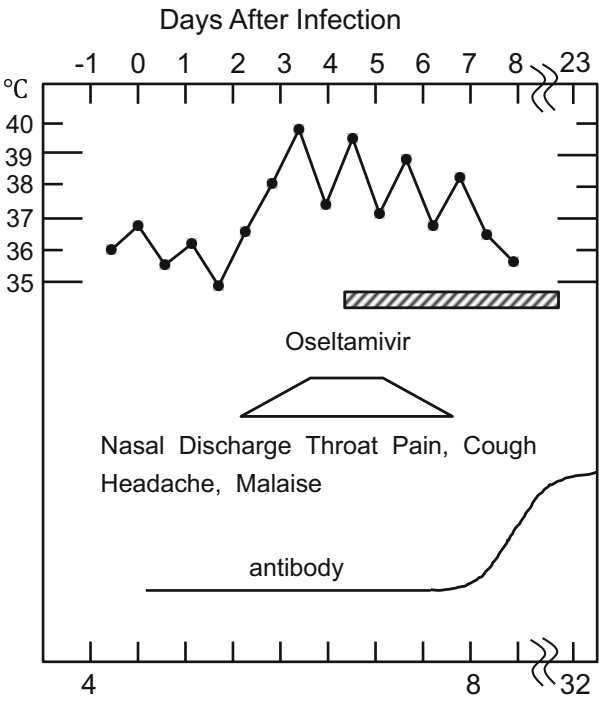
I propose an idea of triangle. It consists of microbes, tissue damages, and inflammation. Unidirectional arrows from microbes to the rest never form a structure. The tight triangle structure of biological significance indicates that any one of the three has bidirectional relationship between the other one of the two and amplification or feedback of signaling can take place. I believe that the simple cartoon shown would give readers a deep understanding of leukocyte function by the idea of interplay after reading this chapter.

Elimination of invading microbes is executed by first-line defense program of innate immunity with both cellular and humoral factors, such as neutrophil, monocyte, macrophage, dendritic cell, complement, and acquired immunity with antibody-mediated events. This book is not a textbook of immunology. I provide a springboard for understanding immunity that would be helpful for further discussion of inflammation and cancer, i.e., common disease influenza.

2.3 Influenza

We have epidemic influenza every winter in Japan [1]. Clinical manifestation includes general symptoms, such as high fever and appetite loss, as well as local ones, such as sore throat (Fig. 2.2). Influenza virus is of 100 nm in diameter and

Fig. 2.2 Clinical course of influenza. Influenza patient was treated by oseltamivir, which inhibits neuraminidase of the virus to death. However, fever persists for several days and antibody against the virus is eventually raised



belongs to the Orthomyxoviridae family. It spreads from human to human in respiratory epithelium through small particle aerosols less than 10 μm by coughing out airway discharges. Three classes are found: A, B, and C. Type A virus, which is prevailing in Japan, has two major glycoproteins on the surface called hemagglutinin (H) and neuraminidase (N), consisting of at least 15 and 9 species, respectively. The Spanish influenza in 1918 that caused pandemic spreading was H1N1, and the Hong-Kong influenza in 1968 was H3N2 [2].

A close bronchoscopic inspection of influenza patients shows mucosal discharge and edema in higher respiratory tract, such as larynx through bronchus. Those are the essential features of inflammation. Histologically, respiratory epithelium of columnar monolayer displays vacuolar degeneration, shrinkage, pyknotic nuclei, and cell loss. The epithelial damage is accompanied by infiltration of lymphocytes and macrophages (see Fig. 1.1 in Chap. 1). The highly pathogenic H1N1 and avian influenza H5N1 cause severe edema, hemorrhage, and leukocyte infiltration in even lower respiratory tracts.

Influenza virus antigens are detected in airway epithelial cells and macrophages. Binding of H to $\alpha 2$ -6 sialylated glycan on the epithelial cells facilitates viral adhesion to the airways. What is next?

Fever is rather common and is caused by PGE2 production in the thermoregulatory neurons in the pre-optical anterior hypothalamus (see Chap. 8 for details). Frequently used antipyretic medicine is non-steroidal anti-inflammatory drug (NSAID) that inhibits COX responsible for PG synthesis. Epithelial cells and macrophages in the influenza virus-infected airways locally up-regulate expression of COX-2 and LOX, which respectively results in the production of PGE2 responsible for vasodilatation and of LTB4, causing TNF α induction and recruitment of neutrophils and lymphocytes. Additional PG CysLT increases vasopermeability (Fig. 1.4 in Chap. 1; see also Chap. 3).

2.4 Influenza and Leukocytes

2.4.1 Cell Death of Infected Epithelial Cells

The adhesion of virions to the epithelial cells is followed by their endocytosis into the cytoplasm. RNA-dependent RNA polymerase of viral but not human origin synthesizes complementary positive stranded RNAs, namely mRNAs, from its genome negative-stranded RNAs. After replication in the nucleus, the viral ribonucleoprotein complex is exported to the cytoplasm. We have shown that an involvement of Hsc70 of host origin in this process [3]. The translation machinery of the host cells is then utilized to generate structural proteins of the virus. Newly formed viruses are subjected to exocytosis after assembly of RNAs and proteins necessary for formation of the virion and propagate to the adjacent epithelial cells. Newly generated host mRNAs are degraded and translation of preexisting mRNAs ceases, which results in cell death of the infected cells. Macrophages in the injured

tissue express TRAIL (see Fig. 8.3 in Chap. 8), which induces apoptosis (see below) of the adjacent epithelial cells. Notably, damage of cells or tissues is an essential hallmark, both a cause and a result, of inflammation.

Readers can see a double-edged sword as an essential feature of host defense mechanism. For example, recruited leukocytes dispose of damaged or dead epithelial cells at the sacrifice of still-alive or uninvolved cells, the discrimination of which can hardly be achieved by the first-line defense program without the presence of highly specific antibody by acquired immunity.

2.4.2 Innate Immunity of Infected Epithelial Cells and NK

The influenza RNAs, which are exposed to the cytoplasm of the infected but still alive epithelial cells, are recognized by pathogen sensors, including endosomal TLR7 and cytoplasmic RIG-I. This activates transcription factor IRF-3/7-mediated production of type I IFN α and β , which activates macrophages and natural killer (NK) cells. IFN β -KO mice are sensitive to influenza infection.

Different from pathogen-targeting antibody produced in acquired immunity that is specific to the pathogens, the first-defense program of innate immunity recognizes structural motifs that are conserved among the molecules pertinent to pathogens. This is generally called pathogen-associated molecular pattern (PAMP) and includes viral RNAs and nonmethylated CpG and bacterial endotoxin or lipopolysaccharide (LPS). Because those molecules also are found in nonpathogenic microbes, another name microbe-associated molecular pattern (MAMP) is proposed. Thus, the sensors are pattern recognition receptors (PRR) and shown in Table 2.1. For example, one of the PRRs, TLR7, can recognize viral single stranded RNAs (ssRNAs) derived not only from influenza virus but also other viruses carrying ssRNA as a genome.

NK cells are derived from their progenitors, which originate from HSC in bone marrow and differentiate in lymphatic organs [12]. Different from B and T lymphocytes, DNA recombination does not take place during their differentiation. Both IL-15 and STAT5 play a pivotal role in their activation. For example, patients with loss of function mutations in STAT5 lack NK cells and therefore are susceptible to viral infection. In response to IL-2, they secrete IFN γ and induce perforin-mediated pore formation in the lipid bilayers of target cells, such as those infected with influenza virus. Through the pores, they cast granzymes to induce cell death.

How NK cells recognize their target cells is rather complicated, because normal cells should not be targeted. NK cells carry reciprocal receptors of opposite function. The negative receptors, such as NKG2A-CD94 hetero-dimers, recognize MHC class I to avoid targeting (see Fig. 1.3 in Chap. 1). If cells lack MHC class I, i.e., down-regulation of ligands for inhibitory receptors, they are allowed to be targeted. On the other hand, the influenza virus H antigen is recognized by the activating receptor natural cytotoxicity receptor (NCR) or lysis-triggering receptor, such as p44 and p46, and perforin-mediated cytotoxicity is induced.

Table 2.1 Pattern recognition receptor (PRR)

	General observations in KO mice	Phenotype of cancer metastasis model in KO mice	Refs.
PTX3	Defective microbial recognition and phagocytosis in PTX3-deficient neutrophils	Reduced tumorigenic and meta-static activity in hPTX3-expressed B16-F10	[4]
CRP	No reduction of atherosclerosis in CRP-deficient mice (ApoE ^{-/-} , LDLR ^{-/-} B6 background)	Not reported	[5]
Complement	Protection from insulin resistance in C1q KO mice	Suppressive effects of rituximab (anti-CD20 chimeric mAb) on the EL4 liver metastasis model are completely abolished in C1q KO mice	[6, 7]
TLR	Increased abnormalities characteristic of SLE, including splenomegaly and autoantibody production in double TLR8/9-deficient mice	Carcinoma-produced versican stimulates IL-6 and TNF production and lung metastatic growth via TLR2/6 in the LLC metastasis model	[8]
NLR	Complement-mediated IL-1b and IL-18 release is dependent on NLRP3 inflammasome	Enhanced NK cell-mediated antitumor responses and suppressed lung metastasis of B16-F10 in NLRP3 KO mice	[9, 10]
CLR	Decreased proinflammatory mediator responses and leukocyte recruitment upon mycobacterial infections in Mincle KO mice	Not reported	[11]

Features as a marker of inflammation in PTX and CRP are shown in Table 1.1 of Chap. 1. The phenotypes of the KO mice are useful to understand the roles played by those PRRs

Innate Lymphoid Cells

The bewildering number of names to define cells of a similar function was focused and uniform nomenclature was proposed that are not linked to the signature cytokines [13, 14]. For example, the well-known NK cells with endogenous cytotoxic activity are the prototype of the group 1 innate lymphoid cells (ILCs), and those without cytotoxic activity but with IFN γ production also are proposed to be included in this group [15]. Nuocytes, natural helper cells, innate helper type 2 cells, and multipotent progenitor type 2 cells are now “group 3 ILCs.” They respond to epithelial cells-derived cytokines, such as IL-33 and IL-25, to produce IL-17 and IL-22 in a transcription factor ROR γ t-dependent manner. Of note, the naming is based on what is produced (ligand) and not what is sensed (receptor) in those cells. This is similar to the naming of neuron in which dopaminergic neurons are those secreting dopamine and not expressing dopamine receptor. In influenza

(continued)

virus-infected lungs, IL-33 produced from lung epithelial cells and alveolar macrophages stimulates the group 2 ILCs, which in turn produce type 2 cytokines IL-5 and IL-13 to induce inflammation but at the same time amphiregulin that is involved in repair of the damaged lung tissues. Group 2 ILCs express IL-33 receptor. IL-33 belongs to IL-1 family of proteins and the system of not only the ligand but also their receptor is rather complicated and will be described in the autoinflammatory disease section in Chap. 8.

TLR7 also activates another transcription factor NFκB, which results in secretion of a variety of growth factors, including IL-1β, IL-6, IL-8, TNFα, CCL2 (MCP-1), CCL5 (RANTES), and CXCL10 (IP-10) (Table 2.2, see in this chapter

Table 2.2 Human chemokines and their receptors. One unique feature of this system is that a receptor has multiple ligands that have been called historically by different names

CC family	
Receptor	Chemokine ligands
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC1)
CCR2	CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), CCL16 (HCC4)
CCR3	CCL11 (eotaxin), CCL24 (eotaxin-2), CCL7 (MCP-3), CCL5 (RANTES), CCL8 (MCP-2), CCL13 (MCP-4)
CCR4	CCL17 (TARC), CCL22 (MDC)
CCR5	CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin), CCL14 (HCC1), CCL16 (HCC4)
CCR6	CCL20 (MIP-3β, LARC)
CCR7	CCL19 (ELC), CCL21 (SLC)
CCR8	CCL1 (1309)
CCR9	CCL25 (TECK)
CCR10	CCL27 (CTACK), CCL28 (MEC)
CXC, CX ₃ , C, and XC Families	
CXCR1	CXCL8 (IL-8) [murine equivalent = KC] , CXCL6 (GCP2)
CXCR2	CXCL8, CXCL1 (GROα), CXCL2 (GROβ, MIP-2α), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6
CXCR3-A	CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)
CXCR3-B	CXCL4 (PF4), CXCL0 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)
CXCR4	CXCL12 (SDF-1), CXCL14 (BRAK)
CXCR5	CXCL13 (BCA-1)
CXCR6	CXCL16 (SR-PSOX)
CX ₃ CR1	CX3CL1 (fractalkine)
XCR1	XCL1 (lymphotactin), XCL2

For example, CXCL1 represents previously called human GROα (growth-regulated protein alpha) that is homologous to murine KC. Human CXCL8 equals IL-8, which is missing in mice, and CXCL1 functions for IL-8 in mice

for TLR). They have chemotactic activity to mobilize blood cells from both bone marrow and lymph nodes. Some of them are just called chemokine to underline the activity. For example, CCL2 secreted from the lung epithelial cells stimulates recruitment of monocytes expressing its receptor CCR2. After entry to the lung tissue, monocytes differentiate to macrophages and dendritic cells (DC). When the macrophages are polarized to M1 to produce NOS and TNF α , pulmonary injury and as a consequence inflammation make a progression.

2.4.3 *Innate Immunity of Macrophages and DC*

Generally, neutrophils come first to attack invading microbes, especially bacteria. Then, macrophages participate in phagocytosis of dead cells that contributed to the battle between invaders and host. Namely, macrophages appear in the resolution phase. DC not only manifests phagocytic activity and secretes proinflammatory substances as macrophages, but also a unique feature of presenting antigens to T cells to exert immune responses. Forty-eight hours after influenza virus infection, central roles of innate immunity are played by leukocytes, such as neutrophils, NK cells, macrophages, and DC.

Morphologically, human DC extends dendrites (see Fig. 2.1 legend for information on mouse DC) [16]. DC distributes from upper to lower respiratory tracts and increases its number after influenza virus infection. Similar to respiratory epithelial cells, DC has pathogen sensors. Therefore, by sensing the virus, it plays a major role in activation of acquired immunity by inducing differentiation and activation of helper T cells (Th). DC has a functional name < antigen-presenting cells (APC) > due to its unique property to present antigens on its cell surface. DC appears to acquire the virus antigens to be presented by direct infection of the virus to the DC and by phagocytosis of infected and dead epithelial cells.

There are two different pathways of DC differentiation from HSC [17]. Langerhans cells in skin and interstitial DC directly differentiate from HSC during the course of development. The terms classical myeloid DC (mDC) and plasmacytoid DC (pDC) are used to describe human DCs that differentiate from HSC in different pathways (mouse DCs are expressed as cDC and pDC).

Monocytes derive from common myeloid precursor (CMP) that originates from HSC and circulate in the blood stream. After entry into tissues, monocytes differentiate into macrophages with extended dendrites. Monocytes also can achieve differentiation into DC in response to GM-CSF and IL-4. This is called monocyte-derived DC (moDC) or inflammatory DC (iDC) that are MHCII⁺ CD11c⁺ CD11b⁺ CD1a⁺. By skipping the monocyte stage, CMP can directly differentiate into CD11c⁺ myeloid DC precursors (pre-type 1 DC) in circulation, which make an entry into tissues to encounter and sense antigens, then migrate to draining lymph nodes to interact with undifferentiated Th cells. Myeloid DCs are subclassified as (1) CD1c⁺ DC (CD1c is an MHC I molecule also called BDCA-1), (2) CD141⁺ DC

(thrombospondin, BDCA-3), and (3) $CD14^+$ DC that is $MHCII^+ CD11c^+$ but lacks CCR7.

On the other hand, lymphoid tissues, such as tonsils and lymph nodes in the upper airway, have $CD11c-CD4^+CD3^-$ cells with morphology, such as plasma cells in the T cell area, which transform to cells with dendrites in response to IL-3 and CD40 ligand (CD40L) (see Fig. 8.3 in Chap. 8 for TNF family, and Chap. 12). This population of cells differentiates from HSC via common lymphoid precursors (CLP) and localize in tissue through circulation as DC precursors (pre-type 2 DC) before being stimulated. They are called plasmacytoid DC (pDC). pDCs are $CD123^+ CD303^+ CD304^+$.

Functional division can be recognized as mDC and pDC are delineated by the expression pattern of TLRs, i.e., mDC expresses TLR1, 2, 3, 4, 5, and 8 mainly for sensing bacteria. pDC presents TLR7 and 9 for virus. Notably, pDC is a strong producer of $IFN\alpha$ and β under viral infection.

mDC produces other key molecules of innate immunity, such as PTX3 and SAP, which can bind H of influenza virus [18]. Different from TLRs that are tethered in plasma membrane or endosomal membrane, PTX3 is a soluble form of PRR released into circulation and free to move. This evolutionally conserved and primitive factor mimics antibody (see Table 1.1 in Chap. 1).

2.4.4 Acquired Immunity by DC, Th, CTL

2.4.4.1 DC and CTL

DC activated by influenza virus migrates to the draining lymph nodes in a CCR7-dependent manner and make differentiation of naïve Th0 cells into effector T cells [19]. Active differentiation and proliferation of T cells and B cells are manifested as lymph node swelling and pain in clinical settings. T cells are classified into helper T (Th) and cytotoxic T cells (CTL).

Cytotoxic lymphocytes capable of killing target cells include NK cells and CTL (previously called killer T cells). CTL is $CD8^+$ and activated through its TCR recognizing MHC class I with antigen on DC. CTL shares the killing mechanisms mediated by perforin, granzyme, and FasL with NK cells that lack TCR. Insufficient cytotoxicity of CTL against virus-infected cells with lowered expression of MHC class I is complemented by NK cells (see Chap. 5). Some populations of both NK cells and CTL become memory cells to defend against viral repeat assaults.

2.4.4.2 Activation of Effector T Cells

Basically, mDC induces Th1 with $IFN\gamma$ production and pDC induces Th2 capable of secreting IL-4, 5, 6, 9, and 13 responsible for B-cell differentiation and growth.

However, both multiplicity and plasticity are found in DC functions. The three-cell model was proposed in the DC-Th interactions [20] (Fig. 2.3).

Signal 1 is binding of MHC class II-antigen complex to TCR, signal 2 is binding of CD28 on Th to CD80-CD86 on DC, and signal 3 is pathogen sensor activation in DC. For example, mDC activated by LPS-stimulated TLR4 produces IL-12p40 subunit that heterodimerizes with IL-12p35 to form fully active IL-12 p75 (signal 3), which polarizes Th to CD4⁺ helper T cells (Th1) that produce IFN γ . The key molecule for differentiation is the transcription factor T-bet that is activated by

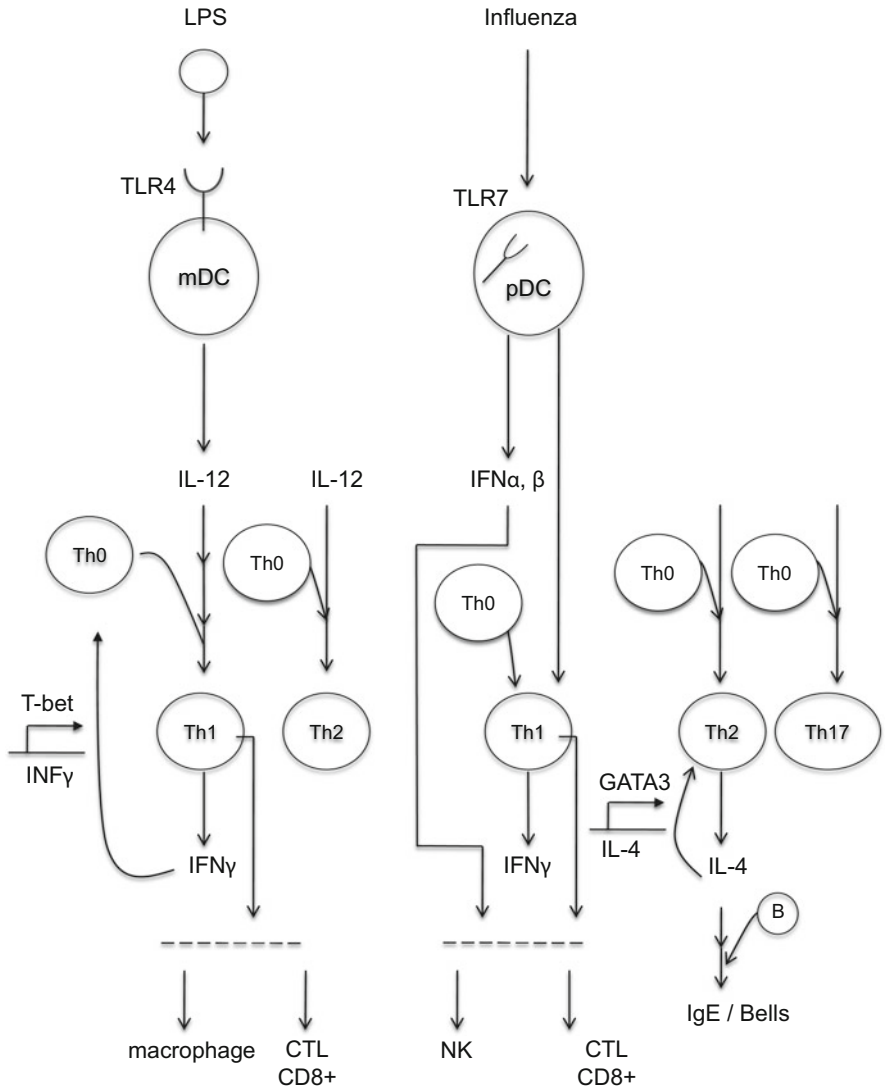


Fig. 2.3 Relationship between DC and Th. See text for details

IFN γ . T-bet also transactivate IFN γ . Then what is the original source of IFN γ ? IFN γ and Th1 are like the chicken and the egg.

One deterministic factor for Th1 polarity is IL-12 levels. IFN γ -stimulated mDC secretes IL-12p35 serving as a positive feedback. LPS from Gram-negative rods, peptidoglycan (PGN) from Gram-positive cocci, and *Haemophilus pylori* are sensed by mDC, which results in IL-12-mediated polarization to Th1. However, Th2 rather than Th1 is induced when mDC is activated by LPS of *Porphyromonas gingivalis* with low levels of IL-12 production. Furthermore, IL-12 expression is suppressed when mDC induces Th2 polarization by histamine or PGE2. You can understand what I have mentioned as multiplicity and plasticity of the DC-Th interaction. Th1 activates macrophages and CTL. In influenza models with mice, intraepithelial CD103⁺ cDC capable of transepithelial migration and access to airway antigens induced CD8⁺ T effector cells.

pDC is less potent for phagocytosis. By sensing influenza virus, pDC secretes type I IFN α that can activate NK cells. In addition, pDC expresses CCR7 in response to influenza virus and migrates towards the T-cell area abundant in its ligand CCL19 (Table 2.2) in draining lymph nodes. pDC induces IFN γ -producing Th1 from CD4⁺ T cells, CTL from CD8⁺ T cells, and IL-4-producing Th2. Th2 differentiation is induced by IL-4-dependent activation of GATA-3, and Th2 activates humoral immunity by producing IL-4. The parasite Helminths are too big to be phagocytosed, and Th2 responses usually take place. Allergic reaction is induced by IL-4, IL-5, and IL-13 produced by Th2 in combination with IgE production and mast cell activation.

2.4.4.3 Th17

DC that participates in phagocytosis of dead cells secretes TGF β and IL-6, synergistic action of which induces Th17 from Th0. Th17 cells are abundant in intestinal mucosa and engaged in IL-17 secretion in a ROR γ -dependent manner and neutrophil mobilization and tissue repair.

When purified naïve CD4⁺ T cells were co-cultured with splenic DC in the presence of TLR ligands, such as CpG or Pam3CSK4, IL-17-producing Th17 cell differentiation took place dependently on MyD88 (a signaling molecule for both TLR and IL-1R; see Fig. 5.3 in Chap. 5) and IL-1R1 in the naïve T cells but not IL-6. This is seemingly contradictory to the alleged notion that TGF β and IL-6 induce Th17 differentiation. The authors claimed that the dependence on IL-6 is guided by DCs in a tissue-specific fashion [21]. The important point is that IL-1 plays an irreplaceable role in Th17 induction. This is supported not only by animal models of IL-1 regulatory molecules, but more importantly, by the actual presence of patients suffering from autoinflammatory syndromes. Lack of IL-1R1 in murine Th1 cells promoted many studies on Th2 differentiation by IL-1 and its implication in allergic disorders. In mice deficient in both IL-1 α and IL-1 β , ovalbumin-induced airway hypersensitivity reaction analogous to bronchial asthma is dramatically reduced [22].

It also was shown that Th17 differentiation could take place in the absence of TGF β , and roles of IL-23 and infected apoptotic cells were proposed [23, 24].

2.4.4.4 Treg

Cytokine storm triggered by a variety of T cells aggravates inflammation and accompanying tissue destruction. The homeostatic role played by Treg is to limit the functions of Th1, Th2, and Th17. A unique feature of Treg is Foxp3+ and its induction requires TGF β . Foxp3-KO mice manifest an autoimmune disorder affecting multiple organs by the de-inhibition [25]. Engineering mice with a given transcription factor knockout specifically in Treg has provided important information on lineage-specific suppression program, i.e., Treg inhibits Th1 through the IFN γ -T-bet cascade and Treg suppresses Th17 via the IL-10-STAT3 cascade.

2.4.4.5 Adaptive

What is the relationship between acquired immunity and innate immunity that serves as a first-line defense? RAG-KO mice that lack both T and B cells are susceptible to influenza infection. (In experiments, human influenza virus of high titers can infect mice via upper respiratory tract but virus production from the infected mice is not high enough to spread to other mice.)

Immunoglobulin IgM, IgA, and IgG against surface glycoprotein H and N started to be produced 2–3 weeks after influenza virus infection and peaked at 4–7 weeks (Fig. 2.2). Class switch of Ig involves IL-4. Antigenic shift that causes mutations in N and/or H raises a serious issue of inability of the antibodies generated against the mutated influenza virus in the future and serves as a fundamental reason why we have influenza every year. Antibody in respiratory mucosa is of IgA type capable of attacking the virus before entry into the epithelial cells. Vaccination has been recommended, because high-aged patients may suffer from severe pneumonia and encephalitis. The vaccines are produced by ether- or formalin-mediated inactivation of the predicted types of influenza viruses that are purified after chicken egg culture. Generation of anti-H antibodies that can confer resistance to infection can be expected by vaccination before prevalence. Whereas the vaccine is usually injected to subcutaneous tissue of forearm, the real infection takes place in the respiratory tract. Whatever the difference in the quality of the virus antigens and route of administration, the virus antigens are processed through DC to Th. Raised antibody is of IgG type but not IgA and therefore circulates in the blood stream instead of being localized in the respiratory mucosa to battle against invading virus. We need 2–3 weeks to obtain the anti-H antibody in our body. Antibody-producing plasma cells are localized in bone marrow and mucosa. Plasma cells are fully differentiated B cells that constantly produce antibody and localized in both bone marrow and mucosa. Both B and T cells can memorize the antigens.

2.4.5 DC and Macrophage Responses to Dead Cells

2.4.5.1 Cell Death

Cell death is categorized as apoptosis, necrosis, pyroptosis, and other proposed modes (Fig. 2.4), and each has its own characteristic features. In necrosis and pyroptosis, intracellular substances appear in the extracellular space to induce inflammation. Inflammation is not induced by apoptosis, in which TGFβ with an anti-inflammatory property is produced. The eukaryotic plasma membrane is asymmetrically organized with phosphatidylserine (PS) only in the inner leaflets. During apoptosis, the PS is exposed on the cell surface, providing macrophages with <eat me> signal. This requires the flippase activity by ATP11C and CDC50A [30].

	Stimuli		Cells	Critical molecules	Morphological features scheme	Ref.
Normal					 Nucleus Endoplasmic reticulum Mitochondrion	
Apoptosis	•TNF •FAS •DNA damage •Growth factor withdrawal		•Any cells (programmed)	•Caspase-3 •Cytochrome c	 •Membrane blebbing and cell shrinkage •PS exposure •Apoptotic body •Chromatin condensation	(26)
Necrosis	•Physical •Chemical •Injury		•Any cells (not programmed)	•HMGB1 release	 •Gain in cell volume (oncosis) •Swelling of organelles •Ruptured plasma membrane	(27)
Necroptosis	•TNF •TLR ligands •Virus		•Any cells (programmed)	•RIPK3 •RIPK1 •MLKL •Inhibited by necrostatin-1	 •Swollen	(28)
Paraptosis	•Trophotoxicity		•Any cells (programmed)	•Inhibited by U0126, DN Nur77, actinomycin D, cycloheximide •HMGB1 release	 •ER swelling •Mitochondrial swelling •Cytoplasmic vacuolation	(29)
Pyroptosis	•PAMPs and DAMPs •Pore-forming toxins •Crystals •UV irradiation	NLRP3	•Macrophages •Dendritic cells •Epithelial cells and possibly other cell types (programmed)	•Caspase-1 •Mouse caspase-11	 Cytosolic swelling Caspase3/7 activation •Membrane swelling •Membrane rupture •Membrane vesicle formation DNA fragmentation Membrane vesicle formation	(31)
	•B. anthracis	NLRP1b		Release : •IL-1β, IL-18 •HMGB1		
	•Flagellin •T3SS •T4SS •DNA viruses •F. tularensis •L. monocytogenes	NLRP4 AIM2		•ATP •IL-1α •LDH		
NETosis	•LPS		•Neutrophil (not programmed)	•Not affected by necrostatin-1 or z-VAD-fmk	 •No morphological signs of apoptosis •Bacteria •NET	(32)
Ferroptosis	•Erastin		•Ras mutant tumor cell (not programmed)	•GPX4 •Inhibited by ferrostatin-1	 •Mitochondrial shrinkage	(33)
Entosis	•ECM detachment		•Breast cancer cell lines (MCF10A, MCF-7) •HEK293 •Primary breast tumor •Prostate cancer cell lines (LNCaP, C4-2) (not programmed)	•Rho •ROCK •Myosin II •LAMP1 •Cathepsin B	 DNA •Death of invading cell •Division •Release of invading cell Cell-in-cell invasion	(34)

Fig. 2.4 Modes of cell death. Approximately 10¹¹ neutrophils undergo apoptosis everyday in a homeostatic manner without inducing inflammation since apoptotic cells show phosphor-serine (PS) exposure as an <eat-me> signal for phagocytes, which is missing in, for example, necrosis that causes inflammation and NETosis of neutrophils that produce ROS and trap bacteria by released chromatin structures that occurs within 1 h after LPS stimulation. Apoptosis, necroptosis, and paraptosis are programmed cell death with different morphological features. Anoikis is apoptosis induced by cell detachment from extracellular matrix (ECM)

In physiological apoptosis of neutrophils at the end of their life span without any phagocytic activity for bacteria, they are phagocytosed by macrophages and DC (see Chap. 14 for detail). For example, PS is bridged by molecules, such as MFG-E8, to $\alpha v\beta 3$ integrin on the phagocytes. This bridging stimulates the phagocytes to produce TGF β , which induces differentiation of CD4⁺ T cells to Foxp3⁺ Treg. Treg further secretes TGF β and IL-10 to brake inflammation. On the other hand, apoptotic cells invaded by Gram-negative rods not only provide the <eat me> signal PS but also exogenous PAMP to DC expressing TLR4. Then, DC produces TGF β , IL-6, and IL-23, collaboration of which promotes differentiation of CD4⁺ T cells to ROR γ ⁺ Th17 cells. Therefore, how the apoptosis takes place determines whether inflammation is induced.

Necrotic cells release intracellular substances, such as nuclear proteins HMGB1 (see Chap. 6), to extracellular space (Fig. 2.4). Pyroptotic cells secrete IL-1 β in a caspase-1-dependent manner. Both HMGB1 and IL-1 β stimulate DC to provoke inflammation as discussed in detail in Part II.

One of the humoral PRRs is C1q [35]. It binds to the dead cell membrane by unknown mechanisms and activates the complement pathway to induce phagocytosis of dead cells by macrophages. Both PTX3 and CRP facilitate C1q binding to dead cells. In the process of complement activation, chemotactic factors, such as C3a and C5a, are generated. Those are called anaphylatoxins and participate in the termination of the inflammation by their chemotactic activity for phagocytes. This is called opsonin effect. However, they also have proinflammatory activities, including histamine release from mast cells and promotion of vascular permeability. This means that they play a dual but persistent role during the course of inflammation. C1q polarizes macrophages to M2 (see below) and suppressed NLRP inflammasome (see Chap. 8 for detail). In general, M2 polarization is required to put an end to a given inflammation.

2.4.5.2 M1 vs. M2

One of the characteristic features of inflammation is the cascade of rather specific transcription factor-dependent expression of cytokines (see Chap. 12 for details) [36]. This regulates the phenotypic switch of macrophages as in the case of T cells.

NF κ B is activated by TLR signaling. STAT1 is activated by IFN β , which is produced in an IRF3-dependent manner [37]. Those activations of transcription factors result in production of proinflammatory substances, such as TNF α and NOS2 by M1-polarized macrophages. M2 polarization is induced by IL-4, IL-10, and glucocorticoid. IL-4 and IL-13 activate STAT6, which results in the expression of M2-polarizing ARG1, MRC1, and Fizz1. M2 macrophages dispose of dead cells (efferocytosis) (Fig. 2.4), and the virus-induced inflammation that is accompanied by injury of respiratory tracts subsides. In patients carrying genetic deletion of complements (see below) in the classical pathway, failure to dispose of dead cells takes place.

2.4.5.3 PGE2

Similar to complements, PGE2 is involved in both inflammatory and resolution phases. PGE2 inhibits the production of proinflammatory cytokines, such as IL-12, IL-6, CCL2, and TNF α , and promotes that of anti-inflammatory IL-10. Suppression of IFN γ and IL-12 by PGE2 gives the Th balance favorable to Th2. The major PG that is synthesized in endothelial cells is PGI2 (prostacyclin) whose analogs, such as iloprost, induce similar anti-inflammatory effects to PGE2 in DC.

2.4.6 Secondary Infection

2.4.6.1 Primary and Secondary Infection

An important feature of inflammation induced by extrinsic factors, such as influenza virus, acquired immunity can prepare for repeat assault. However, at 4 weeks after the primary infection, the inflammation is subsided by innate immunity and repair of the damaged tissues is completed. Prior to the resolution, superimposed infection of bacteria could happen, including *Haemophilus influenza*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. In 1918 when effective antibiotics were missing, the pandemic H1N1 influenza killed millions of people.

Streptococcus pneumonia is wrapped by capsule made of polysaccharides called theca just like peanuts in the shell. Basically bacterial body consists of cytoplasm membrane, peptidoglycan, and cell wall from inner to exterior parts. While the cell wall of Gram-negative rods have lipid bilayer structures containing lipopolysaccharide (LPS) (also called endotoxin) (Fig. 2.5), that of Gram-positive cocci, including *Streptococcus pneumoniae* have C polysaccharide consisting of choline phosphate and ribitolteichoic acid, which is wrapped by theca. C-reactive protein (CRP) reacts with the C polysaccharide, but CRP production is up-regulated in liver in response not to only Streptococcal pneumonia but also to a variety of inflammation induced by other causes.

2.4.6.2 Complement Cascade

C1q binds bacterial membranes. There are three complement activation pathways [35] (Fig. 2.6). Generally, the classical pathway is mainly utilized in response to dead cells while microbes activate the alternative pathway. Theca protects *Streptococcus pneumoniae* against the opsonin effect by complement and killing attacks by leukocyte mobilization. The currently used vaccine for *Streptococcus pneumoniae* is effective and a mixture of an inactivated form of 23 different purified theca membranes. However, there are currently no other vaccines for bacteria.

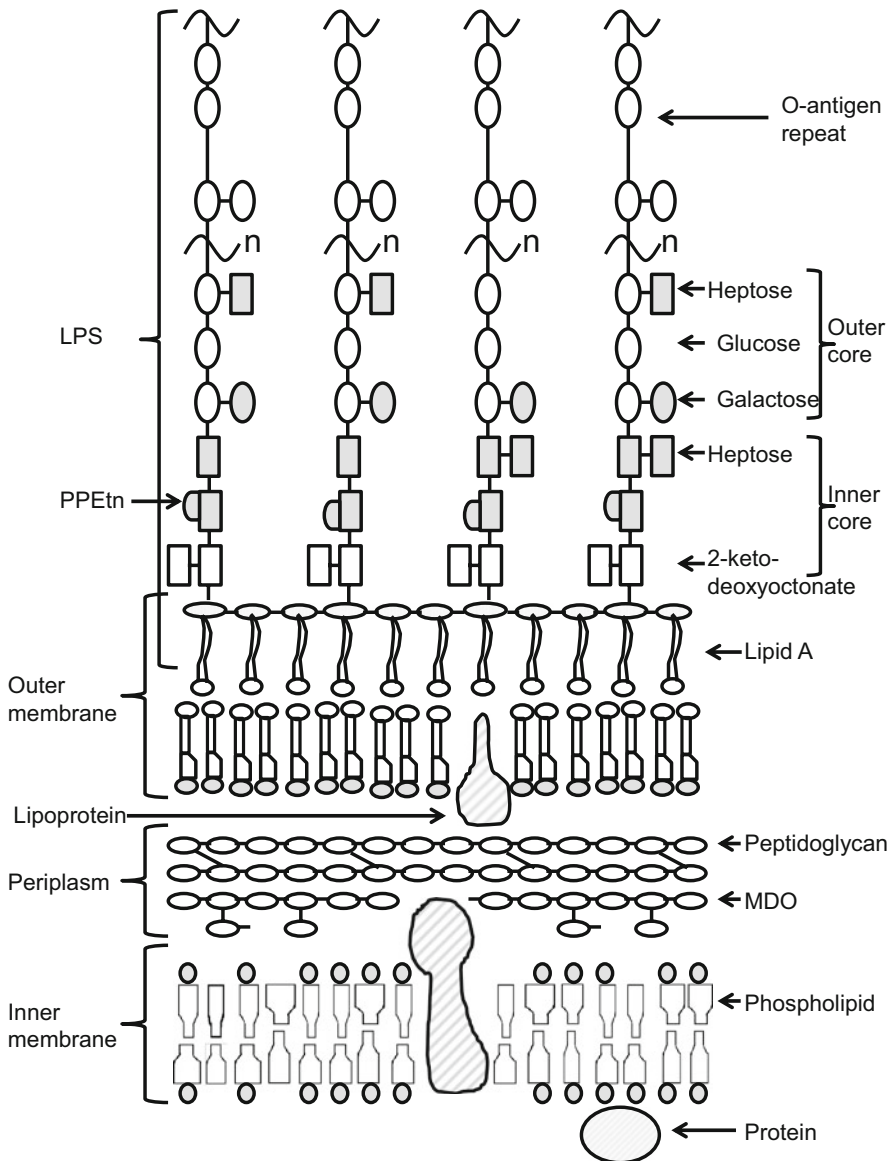
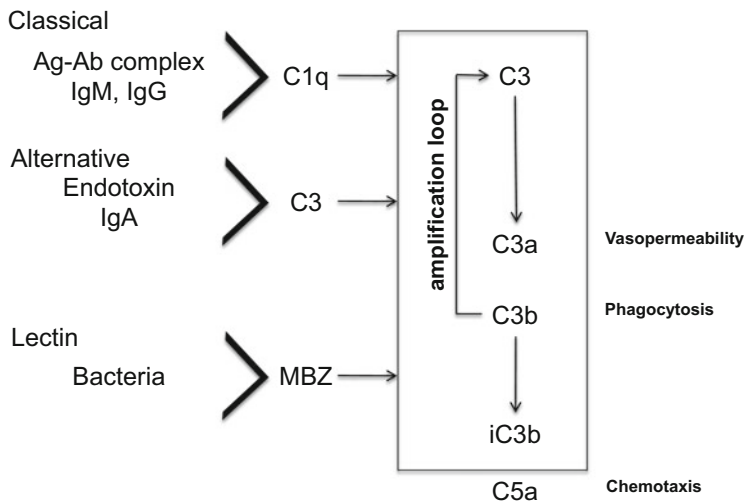


Fig. 2.5 Wall components of Gram-negative bacteria. The most important structure LPS consists of O-antigen repeats, outer and inner cores, and lipid A in the outer membrane. In addition to the cellular arm of the innate immune system as represented by macrophages expressing TLRs on the surface or within the cells, the humoral arm includes complements and pentraxins. Each arm has both sensing and effector functions. *PPEtn* ethanolamine pyrophosphate, *MDO* membrane-derived oligosaccharides



ER-mediated phagocytosis

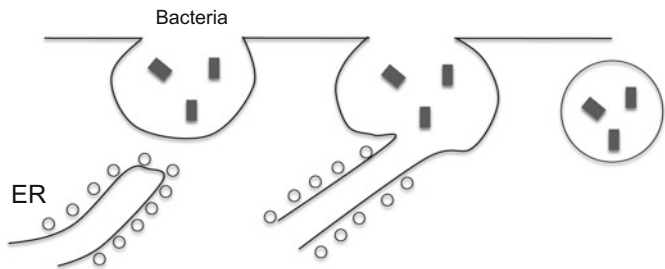


Fig. 2.6 Complement cascade and phagocytosis of bacteria. While C1q binds apoptotic cells, its binding of immune complex activates the classical pathway. A variety of microbial cell surface molecules, such as LPS, bind C3 in the alternative pathway. Mannose-binding lectin (MBL) binds carbohydrate structures of microbes or dead cells and initiates the lectin pathway. Three pathways converge in the subsequent C3 processing. ER is involved in phagocytosis

The three interconnected pathways in complement activation converge in C3 cleavage by pathway-specific C3 convertases. Spontaneous hydrolysis of C3 gives low-level but constant activation of the alternative pathway. The auto-amplification loop of C3 to C3b back to C3 underlies in the core of the three pathways. Biological roles played by the complement system are defense against foreign invaders and clearance of immune complexes, DAMP and PAMP. Mannose-binding protein (MBP) is structurally similar to C1q. C3a and C5a are called anaphylatoxins, and C3b and iC3b have opsonic activity. For example, iC3b binds CR3 (CD11b-CD18) and CR4 (CD11c-CD18), which are integrins responsible for leukocyte migration. Receptors for the anaphylatoxins and C1q cross-talk with TLR4 to enhance IL-6

and reduce IL-12 production [38]. Bacterial wall-bound C3b is recognized by its receptor CR1 (CD35) on phagocytotic macrophages. In the early stage of phagocytosis, plasma membrane underneath the phagocytic cup is fused with ER [39]. This step takes place before proteasome-degraded antigens to be loaded onto phagosomal MHC class I molecules in DC.

2.4.6.3 CRP Receptors

There are several receptors for CRP including Fc γ RII (A, B, C) (CD32) expressed in myeloid cells and endothelial cells [40]. Fc γ R is originally a receptor for Fc portion of IgG. Fc γ R also includes Fc γ RI (CD64), Fc γ RIII (A, B) (CD16), and Fc γ RIV. Structural as well as biochemical analyses revealed that there is no significant difference in the affinity for Fc among the I, II, and III groups. Whereas CD32 is a single polypeptide chain, Fc γ RI, Fc γ RIIIA, and Fc γ RIV have a common γ chain homodimer (FcR γ , pay attention to the similar term Fc γ R; for example, Mincle couples with FcR γ but not Fc γ R) with the intracellular tyrosine-based activity motif (ITAM) as a co-receptor [41]. Through ITAM, tyrosine kinases, such as Syk, Lyn, and Btk, are activated to transmit signaling. As a consequence of IgG-mediated reaction or binding of antibody to antigen, both phagocytosis and cytokine secretion occur. While Fc γ RIIB inhibits through the ITIM (intracellular tyrosine-based inhibitory motif), Fc γ RIIA, Fc γ RIIC, and other Fc γ Rs activate inflammatory responses via ITAM. Mice deficient in Fc γ RII display augmented inflammatory responses to immune complex.

Both CRP and SAP are thought to mimic the IgG function. CRP stimulates not only chemotactic activity but also production of tissue factor (III coagulation factor) (see Chap. 3) in myeloid cells. In endothelial cells, CRP promotes expression of MCP-1 (CCL2), IL-8, and plasminogen activator inhibitor-1 (PAI-1). Thus, the CRP-mediated signaling cascade initiates by activation of innate immunity as exemplified by macrophage activation, which causes both leukocyte mobilization and enhanced coagulation. Disseminated intravascular coagulopathy (DIC) in advanced tumors also has been known for a long time.

What is the biological significance of enhanced reactivity of CRP? As antibody in acquired immunity, neutralizing activity for pathogenic microbes is assumed in CRP. However, interspecies difference between mouse and human has given an obstacle against the CRP research. The levels of CRP response in humans are much larger than that in mice. Given that transgenic mice that were engineered to have the regulatory element in human CRP transcription displayed interspecies conservation in response, it may be possible to examine effects of human CRP in mice. There is a debate on the role of CRP in the resistance against endotoxin shock. SAP is known to bind LPS and assumed to play a suppressive role in the LPS-TLR4-mediated inflammatory reaction (see Table 1.1 in Chap. 1).

2.4.7 Neutrophils

Leukocytopenia in viral infection is thought to be due to suppressive effect of IFN on hematopoiesis in bone marrow. In bacterial infection, mobilization of neutrophils from bone marrow increases their count in the peripheral blood. However, consumption of neutrophils at the inflammatory sites exceeds their production in bone marrow in severe bacterial infection, thus showing neutropenia.

Neutrophils are one of the first-line defenses in bacterial infection. ROS production is responsible for direct bacterial killing. The NADPH oxidase of phagocytes or neutrophils has been renamed Nox2 [42]. Nox2 is a membrane-bound molecule coupled with regulatory molecules, including p47 (also called neutrophil cytosolic factor 1 or Ncf1), p67, p22, and Rac. Activated Nox2 is involved in the catalytic reaction converting oxygen to superoxide in stimulated neutrophils. Generated superoxide is released in the extracellular space. Neutrophils also have phagocytic activity, and phagocytosed bacteria are within the phagosomes. It is of note that the space of the phagosome is originally the extracellular space. Therefore, activated Nox2 in the phagosome can attack bacteria within the phagosome located in the cytoplasm.

Nox is a heme-containing 6-membrane-spanning protein of high genetic conservation from sea urchin [43]. There are five members in humans. Nox2 is classically called NADPH oxidase of leukocytes and tightly complexed to the p22 subunit (Fig. 2.7). Upon various stimuli phosphorylation of p47 cofactor

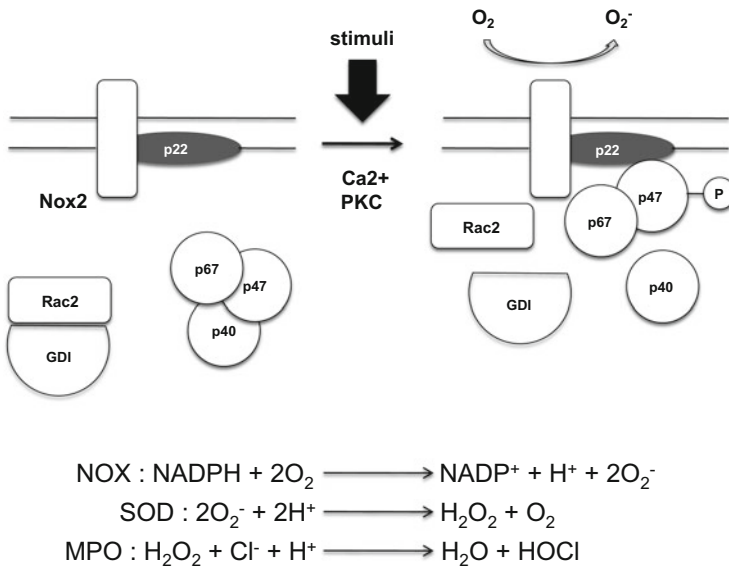


Fig. 2.7 Nox2 activation. Recruitment of co-factors at the membrane activates Nox2 upon various stimuli to generate superoxide, which is then sequentially converted to H₂O₂ and HOCl by superoxide dismutase (SOD) and myeloperoxidase (MPO), respectively

takes place, for example, by conventional PKCs. ROS-induced Ca^{2+} influx into cells further enhances Nox activity. Interactions between p47 and TRAF in $\text{TNF}\alpha$ signaling, Nox2 and TLR2 or Nox4 and TLR4 have been documented. For example, *Mycobacterium tuberculosis* induced a physical binding between TLR2 and Nox2 and TLR2-dependent ROS production is abrogated in macrophages deficient in Nox2. In addition, RIP1-dependent recruitment of Nox1 and Rac1 in the signaling complex of $\text{TNF}\alpha$ -induced necrosis in mouse fibroblasts also was reported [44]. It should be noted that Nox1, Nox2, and Nox4 are responsible of ROS generation in endothelial cells.

Thus, a variety of cellular signals in different types of cells, including those elicited by $\text{TNF}\alpha$ and TLR ligands can produce ROS. Reactions involved in Nox, superoxide dismutase (SOD), and myeloperoxidase (MPO) are shown in Fig. 2.7. Hypochlorous acid (HOCl) is highly bacteriocidal.

Patients with chronic granulomatous disease (CGD) have a congenital impairment in the activity of Nox2 due to genetic mutations in Nox2 or regulatory factors, such as p47, resulting in high susceptibility to infection by *Staphylococcus aureus* that is positive in catalase [45]. Catalase promotes reaction: $2\text{H}_2\text{O}_2 = 2\text{H}_2\text{O} + \text{O}_2$. Normally superoxide spontaneously converts to H_2O_2 , which then reacts with Cl^- to generate HOCl by MPO specifically found in neutrophils. HOCl is a potent bacteriocidal agent but catalase⁺ bacteria can inactivate H_2O_2 derived from superoxide thereby can defend against killing by HOCl.

A polymorphism and natural mutation of p47 are found in rat and mice, respectively. The p47 knockout mice display CGD phenotypes and the polymorphism regulates the severity of arthritis in rat, indicating that Nox2 plays an important role in inflammation by producing ROS in both infectious and sterile conditions. As stated in the influenza sections, oxidized phospholipids cause a cytokine storm, especially IL-6 in a TLR4-dependent manner. Therefore, it is noteworthy that in the absence of bacteria or LPS, the lethality by influenza is dependent on IL-6 and p47-mediated generation of ROS that oxidizes phospholipids in the pulmonary surfactant.

Intratracheal administration of a TLR2 agonist (zymosan mainly consisting of β -glucan from yeast cell wall) in p47-KO mice induced lung exaggerated and persistent lung inflammation with NF κ B-mediated production of $\text{TNF}\alpha$, IL-17, and G-CSF, which was rescued by transplantation of bone marrow from wild-type mice. Mechanistically, anti-inflammatory transcription factor Nrf2 appeared to be impaired in p47-KO mice, suggesting that Nox2 participated in the resolution phase in this sterile inflammation experiment.

Resolution of inflammation (see Fig. 1.1 in Chap. 1) is mediated by phagocytosis of apoptotic neutrophils by macrophages and reverse migration of neutrophils out of inflamed tissues. Kinetic analysis of tissue injury in zebrafish, in which neutrophils and macrophages are separately labeled, revealed that p22-dependent ROS production by Nox2 is necessary for early neutrophil recruitment to the wound and for reverse migration after contact with macrophages [46].

During sepsis, whether in patients or mouse models, neutrophils whose expression is high in Ly6G, CD11b, and $\alpha 3\beta 1$ (VLA-3; CD49c-CD29) (see Fig. 1.2 in

Chap. 1) are observed, which correlates with increased IL-6 and MPO activity suggesting their hyperinflammatory nature [47]. Conditional deletion of $\alpha 3$ in granulocytes resulted in improved animal survival with decreased numbers of extravasating neutrophils.

Neutrophils work in concert with platelets to trap bacteria in extracellular space in a manner dependent on TLR4 on the platelets [48] (see Chap. 3). This is called neutrophilic extracellular trap (NET) (Fig. 2.4), in which PTX3 is known to be co-localized [49]. PTX3 binds KpOmpA, an outer membrane protein of *Klebsiella pneumoniae*, but not LPS or lipoteichoic acid. KpOmpA also is known to activate TLR2.

In addition to Nox2, mitochondrial ROS also contributes to bacterial killing in macrophages. TRAF6, one of the TLR4 signaling proteins (see Chap. 5), is recruited to mitochondria upon LPS stimulation and ubiquitinates ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), which then associates with oxidative phosphorylation complex I components (see Chap. 10), resulting in ROS production in murine macrophage RAW cells [50]. An shRNA-mediated individual knockdown of TRAF6 and ECSIT in bone marrow-derived macrophages dramatically reduced LPS-stimulated mitochondrial ROS generation and concomitant impairment of killing of intracellular pathogens, such as *Salmonella typhimurium*.

2.5 Concluding Remarks

Responses of differentiated effector leukocytes to extrinsic invaders as exemplified by influenza virus are described, including both cellular and humoral arms of immune system. Ideas of pattern recognition receptor, chemokine, and cell death mode are presented.

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Chapter 3

Vessels and Coagulation

3.1 Vessels

3.1.1 Introduction

One of the fundamental features of inflammation is increased vascular permeability. Vessels are anatomically grouped into artery, arteriole, capillary, post-capillary venule, and vein with different structures.

Vessels form a barrier in the absence of inflammation, by which transvascular movement of intravascular substances and cells are tightly regulated in a homeostatic manner. The most inner lining of the barrier consists of a monolayer of endothelial cells. Endothelial cells not only passively respond to substances that are released from leukocytes on inflammation but also actively affect the surrounding microenvironment, including cells and extracellular matrix.

Three essential elements for endothelial cells to participate in the barrier formation are (1) cell-cell adhesion, (2) evasion of cell death, and (3) inhibition of overgrowth (see also Chap. 11). Loss of cell-cell adhesion results in increased permeability through the gaps. Conversely, decreased cell-cell adhesion is frequently observed when vascular permeability is increased. Endothelial cells are thought to have a long life span. Endothelial cells at the bifurcation where blood stream is rigorous are prone to acquire injury, which is counteracted by anti-cell death systems. By contrast, overgrowth of endothelial cells could impair the structural integrity of the barrier by lowering the levels of cell-cell contacts. In this Chapter, the basic elements of the endothelial barrier and its disruption in inflammation are discussed.

3.1.2 Adhesion Machinery

3.1.2.1 Endothelial Adhesion

Electron microscopic (EM) analysis of endothelial adhesion revealed special structures as follows (Fig. 3.1) [1, 2]:

1. Adherens junction (AJ) (also called intermediate junction, zonula adherens, belt desmosome) is formed by homophilic interactions of VE-cadherin in a Ca^{2+} -

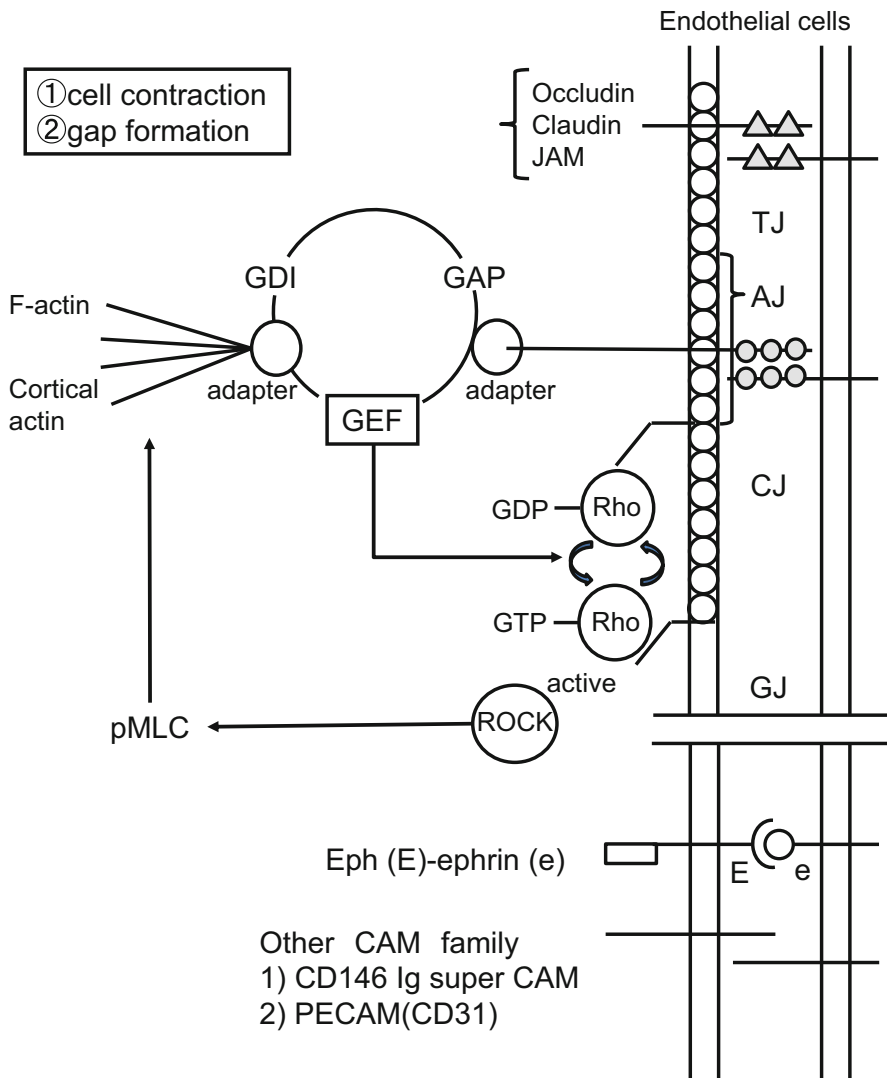


Fig. 3.1 Endothelial cell junctions. Adhesion machinery is regulated mainly by GTP-binding proteins and kinases in the cytoplasm. pMLC: phosphorylated myosin light chain

dependent manner and the gap size is 10–20 nm. β -catenin mediates VE-cadherin and actin.

2. Tight junction (TJ) (also called zonula occludens) is generated by fusion of the outer membrane and involves occludin, claudin, JAM (junctional adhesion molecule), and Nectin-Afadin. Occludin and claudin participate in adhesion of adjacent cells by its homophilic interaction in the extracellular region and by recruitment of intracellular proteins, such as ZO-1, to have connections to actin.
3. Gap junction (GJ) (also called nexus, communicating junction). The size between the cell membrane is 2 nm and substances pass through the tunnels formed by hexameric connexin.
4. Complex junction (CJ) (also called complexus adherens, desmosome-like structures) has an analogous structure to desmosome (also called maculae adherents) in epithelial cells and is found in vein and lymphatic vessels.

VE-cadherin mainly contributes to the formation of AJ, and occludin and claudin to TJ. Different from those molecules whose contribution is rather matched to the morphologically defined structures, the following molecules also participate in endothelial adhesion.

5. CD31 (also called PECAM) is expressed in leukocytes and platelet but not epithelial cells.
6. CD146 (also called S-endo-1, Muc18) is expressed in smooth muscle cells but not in epithelial cells (see Chap. 11).
7. Eph-ephrin is expressed in endothelial cells and partly in lymphocytes.

Generally, cell adhesion molecule (CAM) family consists of the cadherin family, selectin family, integrin family, immunoglobulin superfamily (IgSF). The above-mentioned CD31 and CD146 belong to the IgSF.

3.1.2.2 Caveolae

In addition to the adhesive structures, EM analyses of endothelial cells revealed an omega-shaped invagination of 50–100 nm in diameter called caveolae. One essential hallmark of caveolae is caveolin-1, whose knockout (KO) resulted in loss of caveolae, which could be restored by re-expression of caveolin-1. Caveolin-1 is expressed most abundantly not only in endothelial cells but also in adipocytes and type I pneumocytes. VEGF can cluster caveolae to allow formation of vesiculo-vacuolar organelles (VVO) [3]. Gold-labeled albumin was shown to be transcytosed from the luminal side to the subendothelial space in a caveolin-1-dependent manner.

3.1.3 *Methods for Vascular Permeability*

Herein, I state the experimental methods to examine vascular permeability to evaluate the ability of a given substance (see Fig. 3.2 legend). As I stress everywhere in this book, increased vascular permeability is essential to discuss inflammation.

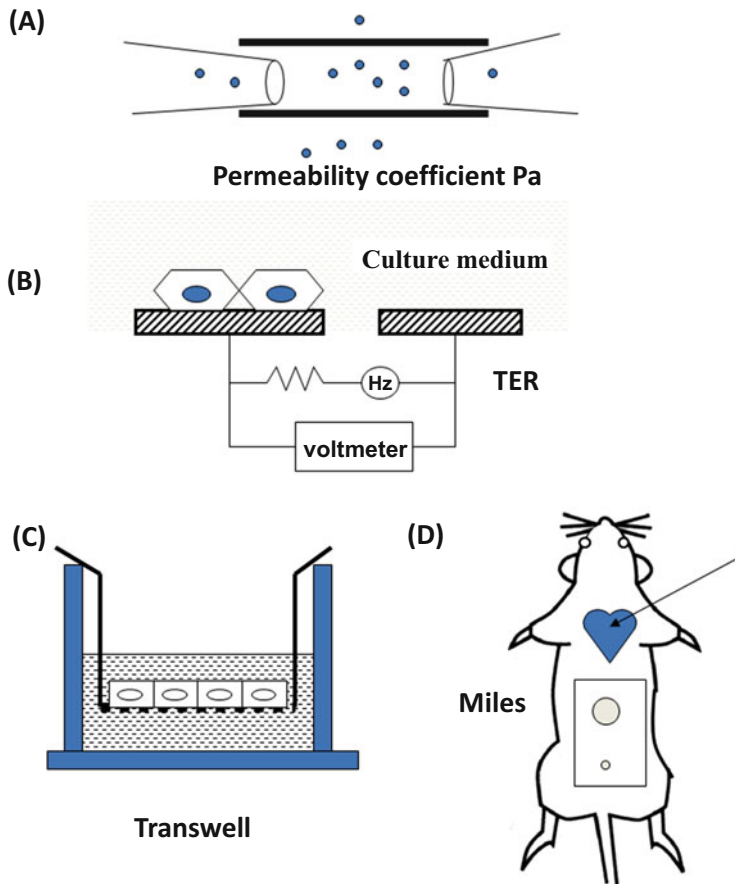


Fig. 3.2 Methods for vascular permeability. Ex vivo assay (a). Capillaries and/or venules of 20–50 mm in diameter and 1 mm in length are isolated and inserted by micropipets at both ends with FITC-labeled BSA-containing solution circulating in the lumen. By measuring physical values, permeability coefficient P_a is calculated. In vitro assay (b, c) [42]. Endothelial cells are cultured on an electrode to measure transendothelial electrical resistance (TER), which depends on the cellular morphology and adhesive property of the stimulated endothelial cells (b). A monolayer of endothelial cells are cultured on Transwell inserts with pores of various sizes (0.1–3.0 mm) and covered by collagen. By putting indicators, such as ^{125}I - or fluorescence-labeled BSA (MW 69 kDa) in the upper compartment, stimulators change the amount of indicators in the lower compartment (c). In vivo assay (d) [43]. Because vessels are basically of in vivo architecture, we need to handle the whole-body. Upon intracardiac injection of Evans Blue (MW 960), which virtually binds serum albumin in guinea pigs, leaked dye at sites of subcutaneous injection of a candidate substance can be semiquantified. This is called Miles assay. FITC-labeled dextran of 40 kDa also can be used

To increase vascular permeability, there should be certain changes in endothelial cells that form the most inner lining of the vessels. Morphological changes of endothelial cells underlie the enhanced permeability in postcapillary venules by

Table 3.1 Permeability factors. Factors that induce vascular permeability

Vascular Permeability Factor	
(*) indirect	
IL-33(*)	PAF
IL-25(*)	Chymase
IL-5(*)	CCL19
IL-13(*)	IL-17
IL-1β	IL-23
IL-6	IL-10
IL-8	TGFβ
TNFα	Complement
CCL2(MCP-1)	C1q
CCL5(RANTES)	C3a
CXCL10(IP-10)	C5a
IFNα	LPS
IFNβ	Ang-2
PTX3	Angptl4
CRP	Fibrinogen
SAP	HGF
IL-4	CXCL12
IL-5	S100A8
IL-6	S100A9
IL-9	S100A4 BBB
IL-13	S100A12
IL-12	Soluble ephrin-A1
IFNγ	SAA3
PGE2	Histamine
VEGF	LPS
PGD2	LTA
LTB4	Angiotensin II
LTC4	

histamine, serotonin, bradykinin, and substance P (Table 3.1). EM analyses revealed that endothelial gaps or intercellular openings are approximately 0.5 μm in size (0.1–3.0 μm) (larger gaps of 1.7 μm as a mean are found in tumor vessels; see Chap. 11). In addition, cell body of a single endothelial cell can display a pore called fenestration. In addition to intercellular opening, VEGF can induce fenestration around 0.6 μm not only in postcapillary venules but also in capillaries. FGF is thought to lack this ability. Summation of pores formed this way could constitute roughly up to 1% of all endothelial barriers. Needless to say, individual receptors for each permeability factor are expressed in endothelial cells.

3.1.4 VEGF

A naturally occurring soluble form of VEGFR1 (sVEGFR1) has tenfold higher affinity to VEGF than VEGFR2 serving as a decoy receptor (Fig. 3.3). VEGFRs are located on both the luminal and abluminal sides of the endothelial lumen, and abluminal ones are predicted to elicit signaling, i.e., VEGF coming from the parenchymal side but not blood. However, in a pharmacological view, VEGF binds predominantly to VEGFR1 with a higher affinity at low VEGF concentrations [4]. Membrane-unbound sVEGFR1 tightly controls the local bioavailability of VEGF to induce bifurcation of vessels. The fact that endothelial cell-specific knockout of VEGF resulted in hemorrhage clearly shows that VEGF contributes to the maintenance of vascular barrier in a homeostatic manner [5].

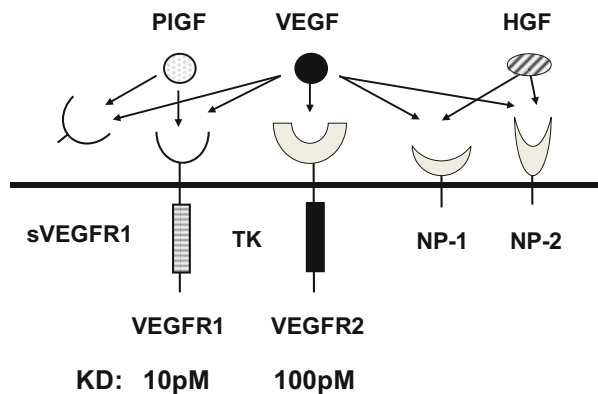
For example, in mosquito-borne dengue hemorrhagic fever, vascular leakage and hemorrhagic symptoms are rather common, which is correlated well with serum levels of VEGF that is unbound to sVEGFR1 [6]. Dengue virus-infected endothelial cells have increased expression VEGFR2. As a consequence, VEGF-induced vascular permeability is increased in this tropical disease.

Tumor cells by themselves express VEGFR1 and VEGFR2, in which VEGF could promote tumor cell proliferation. However, generally VEGF is secreted from tumor cells and surrounding macrophages. Fibrin thrombus formation in cancer that was reported more than 50 years ago is due to hyperpermeability (see Chap. 12).

VEGF-induced VEGFR2 activation signals through at least three pathways. The first is the PLC γ -mediated activation of PKC β , which phosphorylates occludin and ZO-1 to weaken the TJ. More precisely, serine phosphorylation of PLC β 3 negatively and tyrosine phosphorylation of PLC γ positively regulate intracellular Ca²⁺ release, which eventually causes vascular permeability [7].

The second pathway involves Src and FAK activation. The activation of Src is mediated by TSAd (T-cell-specific adaptor)-phosphorylated VEGFR2 at Y951 [8]. Vascular permeability was attenuated in TSAd-knockout mice in Miles assays with Evans Blue or in extravasation of FITC-labeled dextran that was injected

Fig. 3.3 VEGF and its receptor. The simplest scheme for VEGF-A (VEGF indicates VEGF-A in this book) and its receptor. Whereas PIGF specifically binds VEGFR1 and is soluble with a high affinity, VEGF binds several receptors, including NP-1 devoid of the intracellular domain. TK: tyrosine kinase domain



systemically. VEGF-dependent morphological changes in VE-cadherin failed to take place in TSAAd knockout. By contrast, histamine-induced extravasation of fluorescent microspheres was not abrogated in TSAAd knockout. The Src signaling is followed by the activation of the GDP-GTP exchanger VAV2 and subsequent smgRac and PAK activation. Eventually, VE-cadherin is phosphorylated and binds β -arrestin2, thereby promoting clathrin-mediated endocytosis of VE-cadherin and AJ is weakened [9]. Lentivirus-mediated knockdown of β -arrestin2 by shRNAs abrogated the VE-cadherin internalization and VEGF-induced permeability of FITC-labeled dextran in monolayers of SV40-immortalized mouse vascular endothelial cells. Dramatic abrogation of VEGF-stimulated vascular permeability in Src-KO mice indicates the biological significance of this pathway.

The third signaling activates the PI3K-Akt pathway. PI3K makes a complex with molecules associated with AJ and Akt phosphorylates endothelial nitric oxide synthase (eNOS) at serine 1177 to release caveolin-1-mediated suppression of eNOS activity. This is accompanied by translocation of eNOS from plasma membrane to cytoplasm. The activated eNOS catalyzes L-arginine oxidation to synthesize L-citrulline and NO by transferring electrons from NADPH. NO binds its receptor soluble guanylyl cyclase (sGC) to generate cGMP from GTP. PKG (cGMP-dependent protein kinase) is thought to ultimately activate the Erk-1/2 pathway to increase endothelial permeability. Decreased vascular tone correlates with vascular permeability in the VEGF effect. In fact, one of the major side effects of bevacizumab, an inhibitory anti-VEGF antibody, is hypertension induced by increased vascular tone. Increased vascular permeability by VEGF or PAF is impaired in eNOS, but not iNOS, knockout mice. Conversely, caveolin-1 knockout mice displayed increased vascular permeability. For short, in addition to the effects on both TJ and AJ, NO production induces vascular permeability through the Erk pathway presumably leading to pMLC (Fig. 3.1).

3.1.5 Extracellular Matrix

Extracellular matrix (ECM) also participates in vascular permeability. Observed as structures of 40–60-nm thick, ECL mainly consists of collagen, fibronectin, and hyaluronan. The destruction of ECM increases permeability. Active angiogenic areas have endothelial cells that produce ECM. The ECM receptor is called integrin consisting of heterodimeric transmembrane protein subunits α and β . Bidirectional signaling is found between cell and ECM, which regulates cell morphology and motility. The β subunit binds FAK tyrosine kinase, which is tightly associated with another tyrosine kinase Src [10]. Crystal structure analyses have been achieved for both FAK and Src. Both of them are in inactivated states by intramolecular folding in unstimulated conditions. The closed and inactivated FAK is released upon integrin binding and semiactivated by autophosphorylation of 397 (PY397) at the active center. FAK with PY397 then tyrosine-phosphorylates closed and inactivated Src at Y416 for activation. Src with PY416 in turn phosphorylates

Y576/577 of FAK for full activation. This ping-pong mechanism results in activation of both FAK and Src.

A variety of permeability factors, including histamine, thrombin, and VEGF, induce tyrosine phosphorylation of FAK. When the endothelial barrier is intact, integrin binds both ECM extracellularly and FAK intracellularly. Disruption of the ECM-integrin binding or endothelial cell-specific knockout of FAK resulted in increased vascular permeability. Thrombin-induced permeability in anti-FAK siRNA-treated endothelial cells could not be restored in 2 h. Those experimental results indicate that FAK maintains the endothelial barrier in a homeostatic manner and FAK phosphorylation by permeability factors disrupts it.

3.1.6 Temporal Factors in Vascular Permeability

Silver staining of intercellular gaps in endothelial cells has been performed for a long time. Substance P makes a transient silver peak 1 min after administration, which was demonstrated to be gaps between endothelial cells by EM analysis. Histamine also makes them in 2–3 min as judged by TER. VEGF-induced change of Pa (Fig. 3.2) also peaked at 5 min. Subcutaneous injection of VEGF induced fenestration within 10 min. In Miles assay, vascular permeability by histamine and VEGF starts in 1 min and peaks at 5–10 min. Comparative calculation of number of molecules that can induce equivalent levels of permeability between histamine and VEGF showed that VEGF was more than 1,000-fold potent than histamine. Collectively, an important point is that bioactive substances can induce peaks of endothelial permeability abruptly and transiently within a few minutes.

Thrombin-induced vascular permeability displays a biphasic peak, i.e., the first peak within 5 min followed by the second peak of 60-min duration that ends in 120 min [11]. TNF α -induced permeability in HUVEC in Transwell experiments starts after 30 min and lasts for more than 10 h with a peak around 3 h. Under the same experimental procedure, VEGF shows the aforementioned transient peak, which is followed by the second increase after 4 h. Those results strongly indicate the different mechanisms involved in permeability by different molecules. The actual permeability phenomenon in the whole-body should be more complicated than those simplified results, because rates of production, secretion, and disappearance inevitably affect the phenomenon. For example, thrombin is generated by the coagulation cascade (see below), enriched in fibrin clots and gradually released into circulation with a half-life of 5 min.

3.1.7 Common and Fundamental Molecular Biology of Vascular Permeability

Although a variety of molecules are engaged in vascular permeability, the underlying phenomenon includes endothelial cell contraction and formation of intercellular gaps and fenestration. Both AJ and TJ are recruited by intracellular molecules responsible for the maintenance of endothelial adhesion. The major players are small GTP-binding proteins (smg for abbreviation), such as RhoA, Rac, and CDC42, which are anchored to the membrane after posttranslational modification including prenylation [12]. They switch between an active GTP-bound form and an inactive GDP-bound form by regulation of GAP (GTPase-activating protein), GEF (GDP-GTP exchange factor), and GDI (GDP dissociation inhibitor) with a given level of specificity toward each small GTP-binding protein. Those regulatory proteins bind F-actin. Eventual phosphorylation of myosin light chain enhances the interaction between actin-myosin and induces cell contraction. Each vascular permeability factor stimulates its own receptor on endothelial cells to exert intracellular signaling, which acts on the small GTP-binding proteins to activate endothelial cytoskeleton.

I need to underline that smgRhoA and Rac have antagonizing activity and balance between the two smgs controls the endothelial barrier function. For example, because thrombin and HGF activates RhoA and Rac, respectively, combinatory treatment results in a canceling effect on each other.

3.1.8 Other Permeability Factors

3.1.8.1 Overview

There are well-known vasopermeability factors other than VEGF and their mechanisms of permeability induction (Table 3.1). Many inflammatory mediators not only induce leukocyte mobilization directly or indirectly but also have their own receptors in endothelial cells (EC) to induce vascular permeability.

3.1.8.2 Hemorrhagic Injury as Represented by Thrombin

Tissue hemorrhage often is accompanied by severe inflammation. We need to stop passive extravasation of circulatory solutes and cells by patchworks of clot, because destruction of the closed circulatory system may be lethal due to hemodynamic changes. The coagulation system from the standpoint of inflammation is reviewed in the second half of Chap. 3.

It is seemingly paradoxical that thrombin is essential for clot that blocks extravasation, whereas it also can induce increased endothelial permeability to

facilitate extravasation. Similar to histamine, thrombin has its own seven-membrane-spanning receptor coupled with G-proteins called PAR1. PAR1 also induces Ca^{2+} influx, but it can be blocked only 50% by Ca^{2+} chelator BAPTA and pMLC persists. The reason is as follows. PKC activated by the coupled $\text{G}\alpha_{12/13}$ -PLC up-regulated the activity of RhoA-GEF by phosphorylation. Subsequently, activated RhoA activates ROCK. Cell contraction with pMLC is restored to relaxed state with MLC by MLC phosphatase PP1, but ROCK directly phosphorylates PP1 to down-regulate its activity thereby sustaining the contracted state. In short, transient permeability is achieved by Ca^{2+} and sustained by RhoA signaling. In fact, additive inhibitory effects of ROCK inhibitors with BAPTA were observed in thrombin-induced permeability.

3.1.8.3 $\text{TNF}\alpha$

$\text{TNF}\alpha$ causes cell contraction by PKC-mediated RhoA activation and up-regulation of TRPC1 and PAR1. $\text{TNF}\alpha$ also down-regulates expression of JAM-A and occludin at TJ to weaken the cell-cell interaction. Summation of those activities could prolong vascular permeability.

Stimulation of neutrophils by LTB₄, C5a, or KC (CXCL1) resulted in release of $\text{TNF}\alpha$, which was accompanied by vascular permeability in vivo as judged by Miles assays and significantly enhanced in vitro when neutrophils were adherent to ICAM-1 only in the case of C5a and KC. The in vivo effect was abrogated by anti- $\text{TNF}\alpha$ neutralizing antibody or $\text{TNF}\alpha$ -KO background.

3.1.8.4 Complement

In human skin ulcer samples, C1q was localized in the endothelium of the lesion where angiogenesis was active. In Transwell assays of HUVEC with FITC-labeled BSA as a marker, C1q at 10 $\mu\text{g}/\text{ml}$ induced permeability with ERK1/2 activation peaked at 15 min in a boiling-sensitive manner, excluding the possibility of LPS contamination. Wound healing assays in C1q-KO mice revealed that C1q deficiency resulted in less angiogenic reaction, which was rescued by local add-back of C1q.

C5a, a classically known anaphylatoxin, also induces endothelial permeability through Ca^{2+} -mediated signaling to Ca^{2+} influx, PI3K, and ERK. In an orthotopic tracheal allograft rejection model in C3-KO mice, increased endothelial permeability was concomitantly observed with endothelial deposits of thrombin and increased plasma levels of C5a. Administration of a C5a-specific inhibitor NOX-D19 in this condition ameliorated the vasodilatation and increased permeability.

Of note, the discovery of another C5a receptor C5L2 homologous to the original C5aR made the C5aR signaling more complicated. In leukocytes, C5L2 was shown to bind physically C5aR and β -arrestin and is required for C5aR internalization and

signaling to ERK [13]. LPS-induced HMGB1 release from macrophages was impaired in the C5L2-KO background [14]. Whereas C5a enhances LPS-induced secretion of IL-6 and TNF α in circulating monocytes, C5a inhibits the secretion in tissue macrophages. This suggests a switching function of C5a to suppress proinflammatory mediators to avoid the potential tissue damage caused by them and to facilitate resolution of inflammation.

3.1.8.5 Bradykinin and Soluble Ephrin-A1

Bradykinin-activated calmodulin-dependent kinase II phosphorylates serine 1177 of eNOS as in the case of VEGFR-activated Akt. For soluble forms of ephrin-A1, see Chap. 15.

3.1.9 Antagonism in Vascular Permeability

It is usually the case that two antagonizing activities exist in almost all biological events. Here, it is discussed in vascular permeability.

3.1.9.1 Antagonist-1: S1P-S1P1 Maintenance of Barrier [15]

Sphingosine 1-phosphate (S1P) is abundant in serum and released from activated platelet (see below) and mast cells (Fig. 3.4). S1P binds 5 G protein-coupled receptors, S1P1 through S1P5, that differentially signal through hetero-trimeric G proteins and small GTP-binding proteins Rho or Rac. In vessels, S1P1 increases the endothelial barrier intensity by facilitating the VE-cadherin assembly in AJ. The mechanism involves activation of GDP-GTP exchanger called Tiam1 (T-cell lymphoma invasion and metastasis-1) for Rac by S1P1-coupled Gi.

It is of note that activated T cells after proliferation in lymph nodes egress from the lymph nodes by up-regulating S1P1 toward high concentrations of S1P in circulation. A selective S1P agonist, sponimod, is currently in clinical use for recurrent multiple sclerosis in the United States; the drug strongly facilitates internalization and subsequent deficiency of S1P1, resulting in T-cell unresponsiveness to S1P for egress from the lymph nodes and a decrease in the number of effector T cells in circulation toward the target organs brain.

3.1.9.2 Antagonist-2: HGF Maintenance of Barrier [16]

HGFR is a tyrosine kinase receptor for HGF with angiogenic activity and induces PI3K-mediated activation of Tiam1 leading to Rac activation.

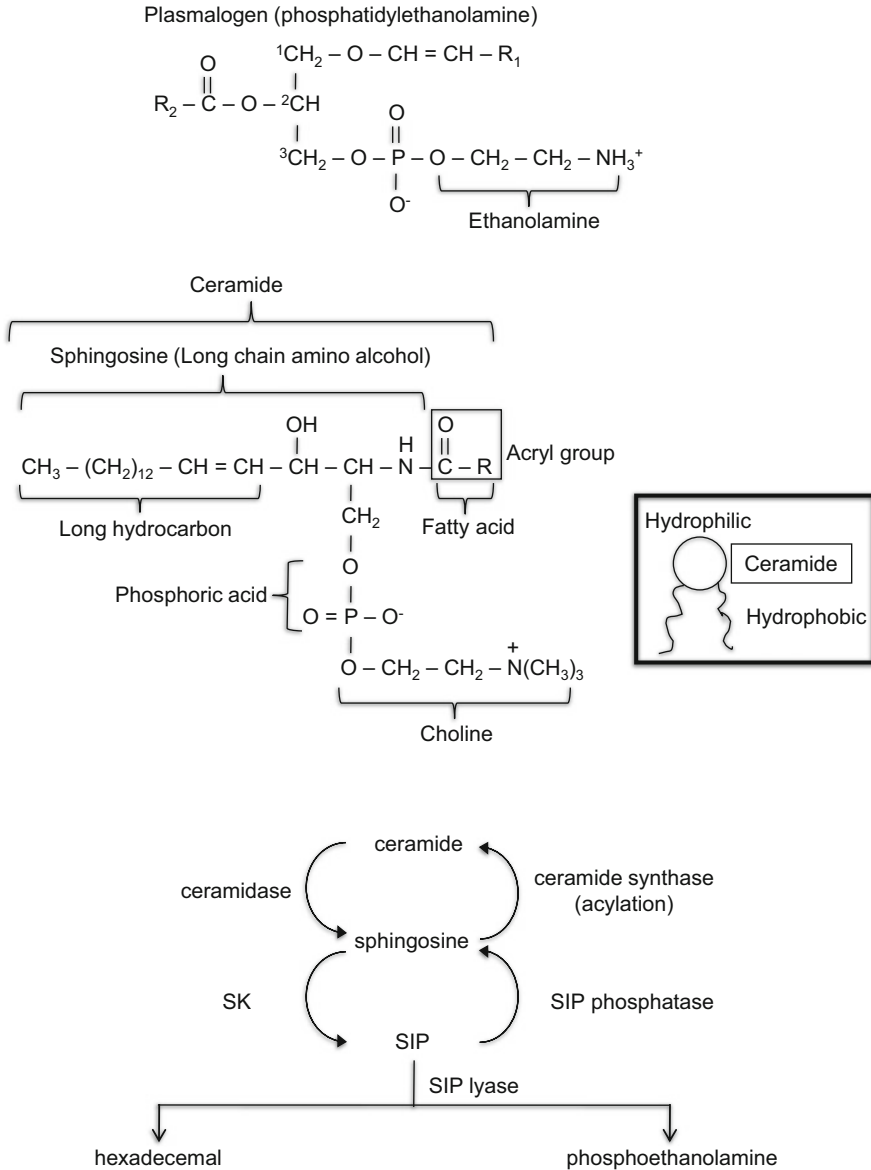


Fig. 3.4 Structure of sphingosine. Basic information on the chemical structures of plasmalogen, ceramide, and sphingosine and on synthesis of sphingosine-1 phosphate is shown. Conversion between ceramide and sphingosine is mediated by ceramidase and ceramide synthase and that between sphingosine and SIP by sphingosine kinase (SK, 2 isoforms are known – SK1 and SK2) and SIP phosphatase. SIP makes an exit from those conversion loops by the action of SIP lyase to yield hexadecenal and phosphoethanolamine

3.1.9.3 Antagonist-3: Angiopoietin 1 (Ang1) Maintenance of Barrier [17]

Given that most permeability factors induce a transient effect, presence of antagonizing activities is assumed. The Ang1-Tie2 signaling retains the RhoA-specific GEF called Syx at TJ. In other words, Syx is required for Ang1-mediated stabilization of TJ. VEGF stimulation activates the VEGFR2 signaling down to PKD-1, which phosphorylates Syx. The phosphorylated Syx is translocated from the TJ to the cytoplasm, thus promoting the junctional disassembly. Monolayers of Syx-KO endothelial cells have altered TER and discontinuity of ZO-1 junctional marker in vitro, which can be only partially rescued by ROCK inhibitors [17]. Syx-KO mice displayed edema and hemorrhage in the interstitial space of myocardium. Extravasation of fluorescent microspheres of 100 nm was more prominent in Syx-KO tracheal venules in vivo. Thus, the shuttling of Syx between TJ and cytoplasm serves as a switch of endothelial permeability.

Notably, there is a second loop of antagonism, i.e., angiopoietin 2 (Ang2)-Tie signaling [18]. Ang2 causes sprouting in vitro. However, Ang2 alone is unable to induce vascular permeability in the tracheal microsphere extravasation assay. Conversely, the permeability induced not only by VEGF but also by histamine or bradykinin was significantly reduced in Ang2-KO mice in the same tracheal assay as well as in Miles assay, which was rescued by adenovirus-mediated expression of Ang2. Loss of Ang2 resulted in reduced PLC γ 2-Ca $^{2+}$ signaling by, for example, histamine.

In summary, while VEGF and Ang1 antagonize at the level of RhoA, endothelial cell-derived Ang2 is likely to work as a paracrine antagonist at the level of receptor Tie2 against Ang1 I in a homeostatic manner.

3.1.10 Aquaporin

Vasopermeability affects cell migration. A local volume change in a single cell is associated with permeability of H $_2$ O and ions, such as potassium. The volume usually expands at the migration front and decreases at the rear for retraction [19]. Aquaporin (AQP) is a tetrameric 6-membrane spanning molecule that selectively can pass H $_2$ O. Endothelial cells express one of the family members AQP1. Hyperosmotic stimuli activate MAPK and HIF-1, both of which induce expression of AQP1. Basically, brain lacks AQP1 expression but tumor vessels associated with brain tumors display increased vascular permeability due to aberrant AQP1 expression. Notably, movement of H $_2$ O is physically associated with cell migration. At the leading edge of angiogenesis, influx of H $_2$ O into the lamellipodia of the endothelial cells serves as a driving force of cell migration under the action-reaction law. It has been reported that tumor growth is inhibited in AQP1 knockout mice with abnormal

vascular morphology [20]. A selective K-channel blocker TRAM-34 could inhibit invasion of glioblastoma [21].

3.1.11 Coupling Biology with Endothelial Permeability

I have repeatedly stressed that vascular permeability and chemotactic leukocyte mobilization are the essential features of inflammation. Most of the permeability factors are indeed chemotactic factors. In addition, permeability is closely associated with coagulation and cell growth directly or indirectly. The most representative factor is VEGF, the most potent factor in angiogenesis in which endothelial cells proliferate by invading into the ECM and differentiating into lumen-forming morphology in a manner directly dependent on its specific receptor VEGFR2. Additionally, VEGF mobilizes myeloid cells from bone marrow through VEGFR1 with a higher affinity than VEGFR2. Furthermore, VEGF is physiologically released from activated platelets during clot formation process. Even in physiological conditions macromolecules go in and out of the endothelial barrier in a tightly regulated fashion, which is disrupted by inflammation. Excess extravasation of disturbed endothelial barrier by whatever reason needs to be stopped by patch works of clot. Thrombin is an essential coagulation factor. $\text{TNF}\alpha$ induces expression of tissue factor (coagulation factor III) through $\text{NF}\kappa\text{B}$ and AP-1.

I propose a triangle of permeability, coagulation, and cell mobilization. The three biological events are interconnected. To explain its functional relationship in inflammation, I refer to the fundamentals of coagulation.

3.2 Coagulation

3.2.1 Definition

Coagulation or clot formation is a process in which blood transforms from liquid to gel. The generated gel is called thrombus or clot. Thrombus formation called thrombosis is usually attached to damaged endothelial cells. Dislocation of the thrombus into circulation and subsequent occlusion of distant vessels is called embolism.

3.2.2 Endothelial Barrier against Coagulation

Normal endothelial cells have intrinsic antithrombotic properties that guarantee smooth circulation. Conversely, damaged endothelial cells rapidly serve a restricted

site of thrombus formation. Clot not only prevents extravasation of circulating solutes and cells but also reserves factors, such as PDGF, VEGF, and TGF β , for the resolution. Therefore, platelets are present in all the phases of wound healing.

Hemostatic plug generation is a complicated event involving platelets, serum coagulation factors, and their modulators and extracellular matrix proteins located in the subendothelial space. Why does the host need this machinery? We are continuously exposed to vascular damages. Deterioration of endothelial walls at the sites of vascular branching by physical forces of blood flow and morphological transformation of endothelial cells by increased expression of vascular permeability factors of inflammation allow movement of small molecules as well as cells through the endothelial barrier. The extravascular leakage needs to be repaired, because it sometimes affects the hemodynamics. The repair begins with elements in the serum that sense the subendothelial collagens exposed to blood. The first-aid is to patch the endothelial openings to block the leakage. In physiological conditions, clot formation must not take place within the blood stream, because it may occlude the vessels to cause thrombosis or embolism. To accomplish the regulation, fibrinolytic system exists to eliminate fibrin gel, the end-product of coagulation, to maintain the vascular homeostasis.

Macroscopic pathology describes three types of thrombus: (1) white thrombus frequently found in arteries, which mainly involves platelet activation and consists of platelets, fibrin, and leukocytes; (2) red thrombus often observed in veins in which fibrin and red blood cells are found; and (3) fibrin thrombus in microvessels.

3.2.3 Basics of Coagulation

3.2.3.1 Coagulation Cascade

The coagulation cascade activated by endothelial injury is the extrinsic pathway initiated by the tissue factor (TF), alternatively called coagulation factor III, released from the subendothelial tissue. The second pathway is the intrinsic or contact pathway and started by XII. The triggering mechanism of XII activation is its contact with anionic surfaces. The activated XII (XIIa) makes an auto-amplification loop, i.e., XIIa cleaves and activates prekallikrein to kallikrein (KK), which activates XII. In addition, kallikrein cleaves and activates kininogen to bradykinin (BK), a well-known endogenous algescic (pain-producing) substance with chemotactic activity for neutrophils. BK causes vasodilatation and vascular permeability. Not only bacteria, such as *Escherichia coli*, but also activated platelets contain and release polyphosphate (polyP) providing a surface for activation of the intrinsic pathway. PolyP failed to induce vascular permeability in BK receptor (B2R) or XII knockout mice as judged by Miles assays.

Both pathways converge at factor X activation (Fig. 3.5). The III–VII complex formation through X eventually leads to the activated thrombin (II). II not only degrades fibrinogen (coagulation factor I) to generate fibrin, the final product of the

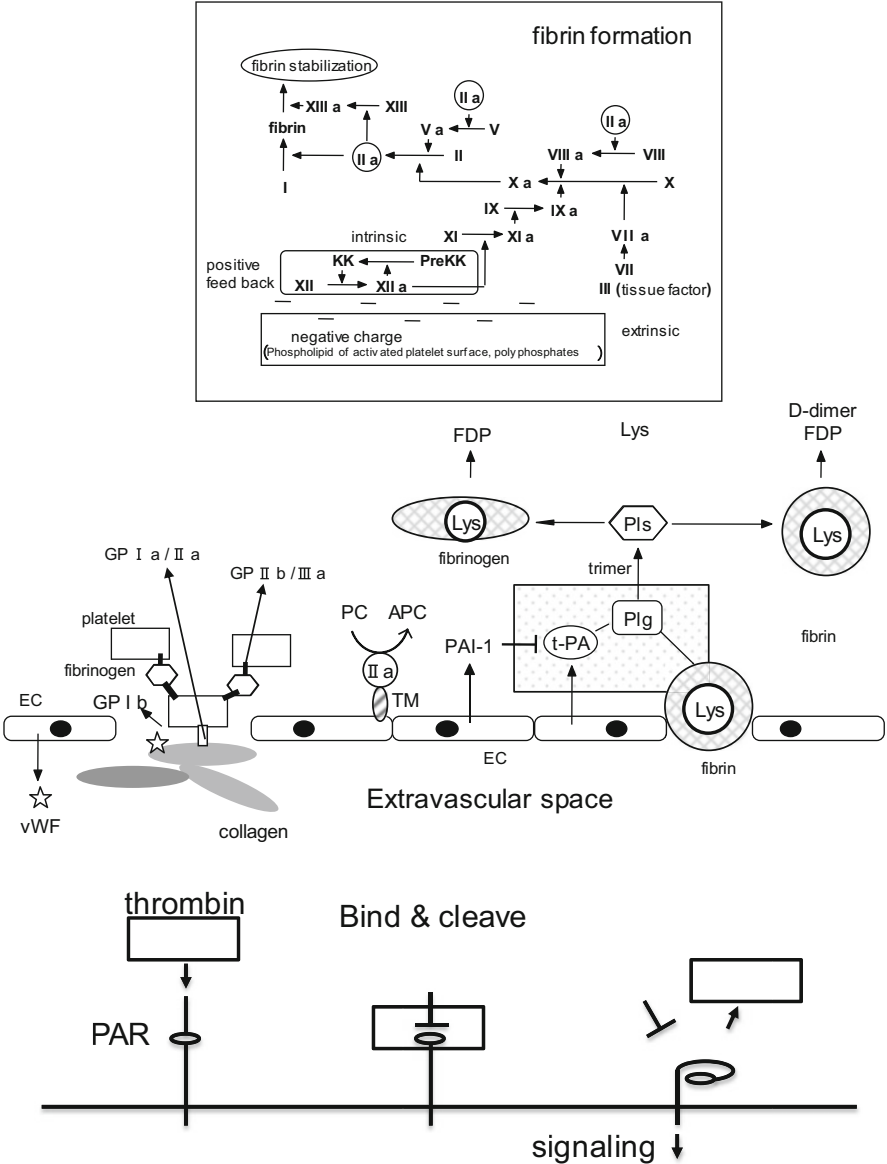


Fig. 3.5 Clot formation and PAR1 activation. Coagulation of both intrinsic and extrinsic cascades is depicted in the top box. Endothelial barrier disruption is fixed by patchworks of platelet aggregation and fibrin clot. Endothelial cell-linked factors involved in fibrinolysis, including TM, PAI-1, and t-PA, are shown. PAR is actually a seven-membrane spanning protein. Thrombin is designated as II

coagulation pathway, but also activates V and VIII for positive feedback or amplification of the coagulation cascade as well as PAR-1 (thrombin receptor) on platelets to induce platelet aggregation via the COX-TXA₂ pathway. Collectively, the coagulation cascade and the platelet aggregation cascade have a reciprocally positive regulation resulting in high production of II.

PAR1-bound thrombin cleaves the N-terminal sequence of PAR1 (Fig. 3.5). The receptor PAR1 contains the domain that binds to itself, but the domain is autoinhibited before the thrombin-mediated cleavage. Therefore, soluble forms of proteases can stimulate PARs with subsequent signal transduction. Of note, fibrin binds plasma fibronectin.

3.2.3.2 Hemolytic Uremic Syndrome

In hemolytic uremic syndrome induced by verotoxin, for example, LPS-bound to platelet can be detected. The activation of TLR4 results in expression of P-selectin, CD40L, and GPIIb/IIIa [22]. von Willebrand factor (vWF) and fibrinogen released from the activated platelet bind GPIIb/IIIa enhancing cross-linkages. Injured endothelial cells release HMGB1, an endogenous ligand for TLR4. Thrombomodulin (TM) was reported to control disseminated intravascular coagulation (DIC) by binding to HMGB1. In addition to endotoxin LPS, metastatic progression of tumor and acute promyelocytic leukemia (APL) sometimes accompanies DIC probably induced by tissue factor (III) expression. DIC exhibits increased coagulation within vessels that consumes both platelets and coagulation factors leading to their prominent loss with propensity to bleed into extravascular space. TLR4 is expressed in not only macrophages but also endothelial cells and platelets [23]. Normally, endothelial cells lack expression of III but LPS-stimulated endothelial cells via TLR4 produce III.

3.2.3.3 Connection Between Platelets and Coagulation

Two major hematopoietic factors, erythropoietin (EPO) and thrombopoietin (TPO), are produced outside of bone marrow, i.e., kidney and liver, respectively. TPO stimulates TPO receptor (also called c-Mpl) on CFU-Meg differentiated from HSC to induce megakaryocytic differentiation through GATA-1. Proplatelets, pseudopodial projections through sinusoidal endothelial cell, are not generated from mature megakaryocytes that are stimulated by TPO. Whereas EPO is produced in response to hypoxia due to a decreased number of red blood cells, TPO production is regulated by marrow stromal cells in response to thrombocytopenia and inflammation in which IL-6 mediates TPO mRNA expression in hepatocytes [24]. However, TPO is trapped by TPO receptors on the surface of platelets and mature megakaryocytes, which may control the TPO levels. Because megakaryocytes replicate DNAs but never undergo cell division, they are multinucleated with 4 N through 128 N. Proplatelets that extend from mature megakaryocytes are eventually

shed to be released as platelets. Approximately 10^{11} platelets are produced daily and appear to be cleared by mechanisms mediated by BCL-2 family members and surface glycans [24]. Platelets carry three types of secretory granules, i.e., (1) lysosome, (2) dense granules containing ADP, 5-HT, and inorganic polyphosphate (polyP) consisting of 60–100 phosphate residues, and (3) α granules with fibrinogen, von Willebrand factor (vWF), coagulation factor V, PDGF, VEGF, IL-6, SDF-1, G-CSF, GM-CSF, M-CSF, and angiogenic inhibitors, such as PF-4, endostatin, and thrombospondin-1 (TSP-1), all of which are released upon platelet activation [25, 26].

In addition to those bioactive molecules contained in granules, I add one more important molecule, sphingosine-1-phosphate (S1P) (Fig. 3.4) [27]. When rat platelet plasma membranes were permeabilized by Streptolysin O (SLO), LDH as a cytosol marker leaked out with ^3H -labeled S1P in conditions where no leakage was detected for makers of α -granule (PF4), dense granule (5-HT), and lysosome (β -hexosaminidase). It is thought that S1P release from activated platelets is mediated by thrombin in an ATP-dependent ABC transporter system, such as ABCC4, and by Ca^{2+} in an ATP-independent manner.

As TPO, many factors involved in coagulation are synthesized in liver. They are proteases in general and activated by proteolytic cleavage. Sequential cleavage forms a cascade, which is regulated by a variety of additional factors, such as liver-derived protein C (PC) and antithrombin, endothelial cell-derived tissue plasminogen activator (t-PA) and vWF, and kidney-derived urokinase-type PA (uPA).

3.2.3.4 Platelet Activation by ECM

Overview

One of the essential events in vascular injury is a direct contact of elements in circulation with collagen on the abluminal side of endothelial cells through the injured sites on the luminal side. Whereas platelets never have adhesive contact with endothelial cells in physiological conditions free of injury due to the action of PGI₂ and NO, both of which are derived from endothelial cells, endothelial injury is recognized by circulating platelets and coagulation factors. PGI₂ induces cAMP-dependent signaling via PGI₂ receptor on platelets, which prevents platelet contact with endothelial cells. Because the physiological role of platelets is clot formation, it cannot be described as <platelets are activated by PGI₂ signaling> unless involved in thrombus formation.

Collagen-Platelet Adhesion

Collagen is recognized by the GPIb-IX-V complex of platelets. Binding of vWF produced from endothelial cells to collagen facilitates the interaction between collagen and platelets. This weak adhesion called tethering allows stronger binding

between them via the GPIa-IIa complex on the platelets. For short, endothelial injury is sensed by platelet and vWF in circulation, which initiates adhesion between collagen and platelets. The contact-activated platelets secrete ADP and 5-HT from dense granules and thromboxane A₂ (TXA₂) after synthesis.

Platelet-Platelet (Aggregation)

Subsequently, contact between platelets called aggregation takes place. The GPIIb-IIIa complex is a receptor for both vWF and fibrinogen from α granules, thereby crosslinking the activated platelets to aggregated forms. Fibronectin binds the GPIIb-IIIa complex and is integrated into clot (notes: clot = blood cells + fibrin = blood – serum, plasma = serum + coagulation factors, blood = blood cells + serum + coagulation factors).

ADP receptor P2Y₁₂ is expressed on platelets and persistent signaling through P2Y₁₂ stabilizes platelet aggregates. The thrombus formed this way is called platelet thrombus or primary thrombus. At the apex of the coagulation, cascade fibrin is formed as described below and participation of fibrin in the platelet thrombus generates the strong secondary thrombus.

3.2.4 *Modulation of Coagulation*

Uncontrolled clot formation could occlude vessels. We have antithrombotic systems to counteract abrupt activation of coagulation.

1. Activated V and VIII are inactivated by proteolytic cleavage by protein C (PC), which is a vitamin K-dependent enzyme synthesized in liver. PC is activated by thrombomodulin (TM) bound to II and exerts its proteolytic activity with protein S (PS) as a cofactor. On the other hand, PC inhibits plasminogen activator inhibitor-1 (PAI-1) [28]. De-inhibition of plasminogen activator (PA) results in plasmin activation. Thus, II induces both positive and negative signaling in coagulation and promotes fibrinolysis.
2. Also produced in liver, antithrombin III (ATIII) inhibits activated II and X around endothelial cells.
3. Plasmin actively lyses thrombus. Fibrin binds both PA and plasminogen (P) and promotes PA-induced activation of P. PA consists of tissue PA (tPA) generated by endothelial cells (EC) and urokinase-type PA (uPA). EC-derived PAI-1 inactivates both PAs to suppress fibrinolysis. Alpha₂ plasmin inhibitor (α 2PI) inactivates plasmin. Congenital defect in either PAI-1 or α 2PI displays enhanced fibrinolysis, i.e., clinical difficulty to stop bleeding in injury.

3.3 Triangle of Angiogenesis, Coagulation, and Inflammation

3.3.1 Overview

The idea is not a chancy one as often misled by a vague picture that VEGF and IL-6 equal angiogenesis and inflammation, respectively, and VEGF induces IL-6 thereby putting an arrow between the two. Those three are independent and any two of those three biological events affect each other in a biological sense with molecular terms being used to explain the relationships (Fig. 3.6). To repeat my definition of inflammation, vascular permeability and cell mobilization are necessary and sufficient for inflammation.

3.3.2 Angiogenesis affects Coagulation and Inflammation

It is clear that VEGF is the most essential and potent factor in angiogenesis. VEGF not only stimulates new proliferation of endothelial cells from preexisting ones but also was originally identified as a vascular permeability factor. Two arrows go from VEGF to angiogenesis and inflammation. This is not what I intend to do. Because many molecules are involved in angiogenesis, as I stated before, I use a few representative molecules for simplicity.

One educational experiment by Harold F. Dvorak in 1979 showed that quote <within a few hours of implantation in the subcutaneous space of strain two guinea pigs syngeneic line ten tumors became invested in a fibrin-gel> unquote [29, 30]. Later, it was revealed that the line ten hepatocarcinoma produces VEGF capable of inducing thrombosis or fibrin clot formation in the skin. When injected into peritoneal cavity, fibrin was observed in the peritoneal wall even with apparent absence of insoluble fibrin in the ascites. High dosages of VEGF from tumor cells destabilize or even destroy endothelial barrier, which activates the coagulation system. Importantly, physiological dosages of VEGF, on the contrary, protect the endothelial barrier by an autocrine mechanism. Genetic deletion of VEGF specifically in endothelial cells in VE-cadherin-Cre/VEGFlox/lox mice exhibited microhemorrhage, intravascular thrombosis, and microinfarctions by 25 weeks of age with endothelial degeneration but no changes in circulating and tissue levels of VEGF [5]. It can be said safely that endothelial barrier is disrupted by either pathologically active angiogenesis with high VEGF levels and by deficiency of VEGF acting directly on endothelial cells, both of which result in extravasation of blood components leading to activation of coagulation system.

High levels of VEGF are detected in lung tissues of asthmatic patients in which Th2 immune responses are believed to play a major role (see Chap. 2). In conditional transgenic mice in which VEGF was induced specifically in the lung club cells by their CC10 promoter in an external doxycycline (dox)-regulated manner,

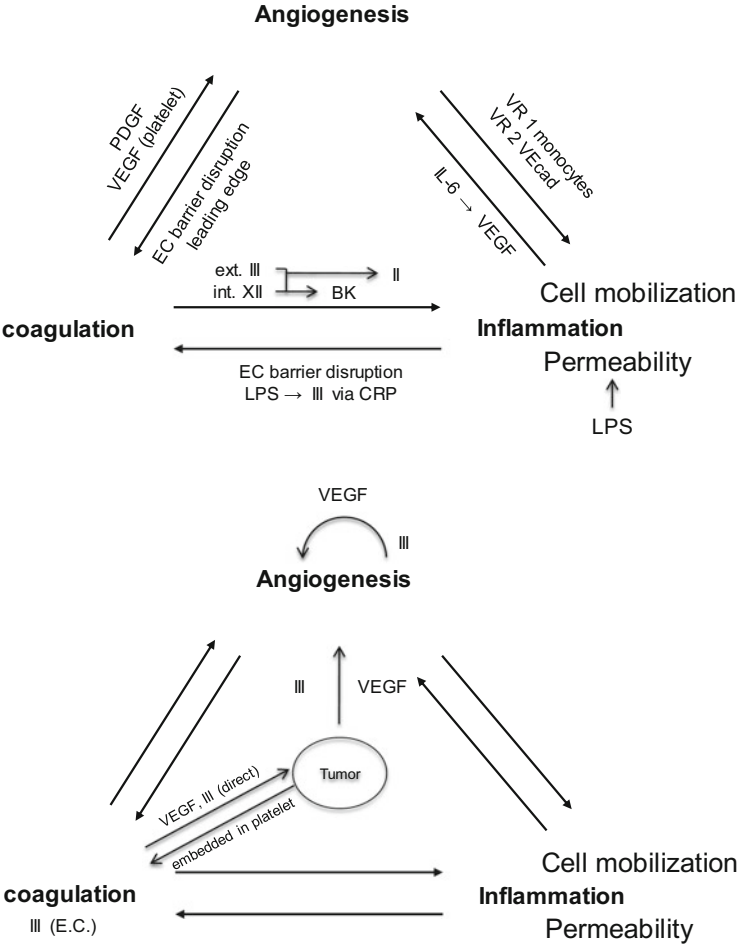


Fig. 3.6 Triangle. In the triangle, any two of the three, i.e., angiogenesis, coagulation, and inflammation, bidirectionally affect each other. For example, VEGF induces III in endothelial cells and III promotes VEGF production in fibroblasts [44, 45]. The original idea was described in reference [46] by the author (see Chap. 12 for an application of the idea to tumor)

Th2 inflammation took place, including myeloid cell infiltration 2 days after dox that is followed by that of both CD4⁺ and CD8⁺ T cells, mucous metaplasia, neovascularization with increased vascular permeability, increased numbers of CD11^{high}-MHCII^{high}CD11b⁺ activated DC, and increased expression of both TGFβ and IL-13 [31]. Although IL-13 could stimulate VEGF expression, the responses were reversible when VEGF expression was stopped by dox-free drinking water. Crossing the mice with those deficient in IL-13-KO revealed that only mucous metaplasia among the above-mentioned features was dependent on IL-13. Ovalbumin aerosol-induced sensitization as monitored by OVA-specific IgG was

dramatically induced. This experiment clearly indicates that VEGF can link between angiogenesis and inflammation.

In addition to the above-mentioned phenomenon caused by VEGFR2, VEGF induces mobilization of monocytes expressing VEGFR1. In a hypoxia-induced angiogenesis model of hindlimb ischemia, the angiogenesis required recruitment of bone marrow-derived CXCR4⁺ cells (BMDC) to the sites of neovascularization. The BMDC recruitment is impaired in knockout mice of vesicle-associated membrane protein-8 (VAMP-8, also called endobrevin) defective in secretion of α -granules but not pearl mice deficient in dense granule and lysosome secretion [32]. In addition, infusion of platelets from TSP-1-KO mice showed enhanced recruitment of BMDC in a tumor implantation model. This experiment indicated that neovascularization is accompanied by platelet activation, which induces leukocyte mobilization in the angiogenic sites.

3.3.3 Coagulation Affects Angiogenesis and Inflammation

As stated, clot formation is accompanied by activation of both platelet and coagulation cascades whether intrinsic or extrinsic. XIIa-activated BK in the intrinsic pathway, and the most essential factor of both pathways, thrombin, designated as II, can induce vascular permeability. Thrombin also is chemotactic for monocytes. Platelet activation releases a variety of bioactive substances, among which both PDGF and VEGF participate in angiogenesis for repair of the damaged tissue.

In the clotting system, thrombin induces activation of PAR, fibrinogen to fibrin conversion, and XIII-mediated fibrin stabilization. Prothrombin-KO mice showed lethality in both embryonic and neonatal periods due to hemorrhage. However, apparently clotting-unrelated substrates are also digested by thrombin, including protein C, complement C5, and osteopontin, suggesting its involvement in a wide spectrum of biological events [33]. In poly (I:C)-inducible ablation of the thrombin gene in liver, conditionally rapid and nearly complete loss of prothrombin took place in circulation. In addition to bleeding events, which were found in heart, skeletal muscle, skin, and brain, a prominent inability to eliminate intraperitoneally injected *Staphylococcus aureus* was observed.

I introduce another nice experiment to prove that coagulation can be located upstream of inflammation by Wolfram Ruf group in 2008 [34]. LPS-induced septic shock frequently causes intravascular coagulation, which can be reasonably rescued by genetic knockout of III, VII, or PAR1 because of the activation of the extrinsic pathway ending in the II-PAR1 signaling. Multiplex cytokine profile of LPS-induced responses displayed PAR-1-dependent induction at 18 h but not 6 h, including IL-1 β , IL-13, IFN γ , and VEGF. The inflammatory responses also were impaired in sphingosine kinase 1 (SK1) knockout (Fig. 3.4) [35].

How we can demonstrate which comes first? If an augmented B signal can give the same phenotype in A-deficient condition as in the condition under proficient A and B, it safely can be said that B is downstream of A. The phenotype is the

cytokine induction responsible for lethality. An agonist for both S1P1 and S1P3, but not S1P1 alone, reversed the phenotype in the PAR1 knockout, indicating that S1P3 is located downstream of PAR1. Although coupling between PAR1 and S1P has been shown in endothelial cells, they found that transplantation of bone marrow of wild-type mice were sufficient to rescue the phenotype of PAR1-KO mice. Dendritic cells (DC) express both receptors as well as III. S1P3-KO mice with adoptively transferred PAR1-KO DC showed LPS-induced lethality accompanied by fibrin clot in mesenchymal lymph nodes only when they were pretreated by an S1P3 agonist. Those well-designed experiments clearly showed that II, the key product of coagulation cascade, acts on DC to exert S1P3 signaling resulting in inflammatory responses.

Fibrinolysis is regulated by plasminogen (Plg) binding to its receptors (Plg-Rs) on the cell surface where plasmin generation is accelerated. Several Plg-Rs have been identified including S100A10 complexed to annexinA2 [36]. Phagocytosis by macrophages is not only promoted by Plg addition but also dependent on Plg. Phagocytosis in vitro of fluorescently labeled apoptotic thymocytes by J774A.1 macrophage-like cells was augmented by 3.7-fold by pretreatment by Plg for 24 h in a manner dependent on plasmin activity. When the same prey cells were intravenously injected into Plg-KO mice, their clearance in spleen was significantly delayed [37]. Coagulation and subsequent fibrinolytic pathways are tightly linked to innate immunity.

Although the precise mechanism is still unknown, an interesting experiment to discriminate functions between soluble fibrinogen and insoluble fibrin was shown [38]. The thrombin cleavage site in the A α chain of fibrinogen was mutated (FibAEK), which disabled fibrinogen from releasing fibrinopeptide A and from inducing clot formation due to inability to polymerize without affecting interaction with platelet for aggregation in vitro. Clearance of intraperitoneally injected *Staphylococcus aureus* was severely impaired in the transgenic FibAEK mice (fibrinogen + polymer-) but not completely absent as in the case of fibrinogen-KO mice (fibrinogen- polymer-), suggesting that fibrin polymer is critical for antimicrobial defense.

3.3.4 Inflammation affects Coagulation and Angiogenesis

Microbe-derived exogenous molecules and endogenous mediators induced by them both can activate coagulation and angiogenesis directly or indirectly, depending on the intensity of proinflammatory cytokine-induced damages to endothelium. For example, whereas LPS directly induce vascular permeability, LPS-induced CRP can promote expression of III to cause coagulation indirectly and LPS-induced IL-6 further causes VEGF expression.

As stated above, platelets express TLR4, its coreceptor MD-2 and an essential signaling protein MyD88 [22]. LPS directly stimulated washed human platelets to induce ATP from dense granules and P-selection from α granules. This

phenomenon was not observed in platelets derived from TLR4-knockout mice. LPS potentiated platelet aggregation and promoted FeCl₃-injured carotid artery thrombus formation in a manner dependent on MyD88. In addition, LPS-stimulated platelets derived from wild-type but not platelet-specific knockout mice released serotonin, known to be essential for vascular remodeling with smooth muscle cell growth in pulmonary hypertension [23]. Hypoxia induced pulmonary hypertension in mice deficient in TLR4 specifically in myeloid cells but not platelets. In a TNF α -induced venular inflammation model, adhesion between platelets and neutrophils were impaired when either Nox2-deficient platelets or neutrophils were infused into the model mice. Mac-1 (= α M β 2 integrin) binding to GPIb-IX-V mediated the heterotypic interaction between platelets and neutrophils, suggesting a regulatory role of Nox2-generated ROS in the binding [39].

Another example is PTX3 (see Table 1.1 in Chap. 1 and Table 2.1 in Chap. 2). PTX3 is mainly produced in macrophages via TLR signaling through MyD88 (see Chap. 5). In both skin wounding and acid-induced lung injury models, excessive and persistent deposition of fibrin was observed in PTX3-KO [40]. Enhanced colocalization of PTX3 with plasminogen and fibrin was observed in the damaged dermis of lower pH in skin wounding compared with undamaged skin, and PTX3 bridged between fibrin and its protease precursor plasminogen *in vitro* in a pH-dependent manner with mean K_d values 7.5 nM and 66.7 nM, respectively, at pH 6.0. Recent evidence showed that PTX3-KO de-inhibited Factor H-regulated complement activation, resulting in C5a accumulation with subsequent induction of CCL2 expression [41]. Administration of anti-CCL2 blocking antibody completely reversed 3-MC-induced sarcoma development in PTX3-KO mice. This indicates that PTX3 plays a homeostatic role in inflammation by mitigating the overshoot of CCL2 via complement [41].

3.4 Concluding Remarks

Endothelial cells serve as barriers against both intravascular clot formation and leakage into extravascular space, which is regulated by adhesion molecules and a variety of permeability factors. Angiogenesis, coagulation, and inflammation are tightly interconnected and form a functional triangle.

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Chapter 4

Sterile Inflammation

4.1 Sterile Inflammation

As stated in Chap. 1, we define inflammation as tissue conditions that accompany two hallmarks: leukocyte recruitment and vascular permeability. It is worth repeatedly stating that chemokines or growth factors that stimulate cell migration also can serve as vascular permeability factors (Table 3.1 in Chap. 3). Readers should remember the three essential elements of inflammation; cause, space, and time.

I briefly covered white blood cells, vessels, and coagulation in Chaps. 2 and 3. The last focus is on resident cells in tissues or organs. Suppose a certain tissue is destroyed by some causes, for example sterile wound; the tissue resident cells are the most certain victims of the injury.

In this Chapter, I will describe inflammation caused in a sterile condition—in other words, in a perfect absence of microbes as represented by tissue injury by irradiation. Secondary infection that may follow the primarily sterile inflammation should be excluded, because the originally sterile inflammation by itself accompanies tissue destruction that may allow microbial invasion. Absolute or relative deficiency in the complement system (see Chap. 2 for details), which can scavenge the cell debris, results in failure of the disposition of dead cells. The pathological persistence of cell debris is to activate immune response, including the production of autoantibodies by B cells. The autoantibodies attack the antigen-presenting resident cells in the tissue or organs, leading to further damage. In those cases, molecules derived from microbes, including nucleic acids and lipopolysaccharides, never participate in the induction of the inflammation, at least in its triggering mechanisms, although secondary infections by microbes may aggravate or prolong the initially sterile inflammation. Therefore, the molecular events are expected to be analogous to or even the same as pathogen sensor-mediated cellular signaling.

4.2 Phenomenological Research

4.2.1 Irradiation

One of the absolutely pathogen-free ways of tissue injury is irradiation, including X-ray, ultraviolet (UV), and laser. Both X-ray and UV can modify DNAs and also proteins. The damaged DNAs are sensed by several sensor proteins and subsequently subjected to repair by arresting cell cycle. Large, in other words, un-rescuable DNA damages result in cell death.

Acute X-ray irradiation induces gross effects in organs where cell turnover is active, such as bone marrow and intestine. In both cases, stem cells pertinent to the organ may remain arrested at G0 and progenitor cells that give rise to mature functioning cells may die. In the intestine, epithelial barrier is therefore abrogated, allowing bacterial invasion. Before microbial invasion, vessels are dilated and cell death of endothelial cells also is found.

It is known that UV induces gene expression of IL-8, a well-known chemokine. Even a low dosage of ultraviolet (UV) induces erythema. This is one of the pathological features of inflammation. Severe sunburn can even lead to blistering and exfoliation of the skin. Although UV induction of Treg, which has a regulatory or inhibitory role in immune responses, has been reported, virtually no secondary infection of the skin after sunburn or UV-treated dermatological disorder, such as psoriasis, implies a presence of tight defense mechanisms in dermal tissues. Ultraviolet induces up-regulation of antimicrobial peptides, such as psoriasin (alternative name = S100A7), human β defensins (HBD), RNase-7, and so forth in the epidermal keratinocytes. Those small peptides of host defense count more than 1900 in the online database and constitute one of the primitive arms of the innate immune system, including TLR, complement, and pentraxin. It seems that most of the documented antimicrobial peptides have pleiotropic activities. HBDs can trigger release of autacoids, such as PG and histamine, and serve as a chemoattractant. A homolog RNase-5 with another name, angiogenin, is involved in angiogenesis. All of the information suggests a role of antimicrobial peptide in UV-induced inflammation.

The enhanced skin responses with pathological outcomes, including severe dermatitis and skin cancer progression, are observed in xeroderma pigmentosum (XP) patients. XP is a rare autosomal recessive disorder as initially noted by the skin lesion during infancy due to hypersensitivity to ultraviolet (UV) with the wavelength ranging from 290 to 320 nm (UVB) (note; UVA 320–400 nm, both UVA and UVB are contained in sunlight that reaches the earth. UVA penetrates more deeply into the skin than UVB). The genes involved in nucleotide excision repair are impaired, and the patient cells can hardly fix the DNAs distorted by UV-induced lesions, such as pyrimidine dimers. Cell death in those cells with irreparable DNAs results in dermatological symptoms, including erythema, bullae, scaling, telangiectasia, and freckles.

4.2.2 SLE

Photosensitivity is one of the most frequent clinical manifestations and the criteria in the diagnosis of systemic lupus erythematosus (SLE) of unknown etiology. The UV-aggravated skin lesion can remain even for months. Dermatological histology includes vacuolar degeneration of basal keratinocytes in the epidermis and lymphocytic infiltration in the dermis. Immunoglobulin and complement deposition are observed in the junction between epidermis and dermis. The high frequency of the presence of antinuclear antibody in SLE indicates the autoimmune nature of SLE. Therefore, UV may aggravate the immunologically induced sterile inflammation in the skin of patients. A difference in the target tissue contributes to the occurrence of Guillain-Barré syndrome as described below.

4.2.3 Immunity-Mediated Inflammatory Disorders

4.2.3.1 Guillain-Barré

The acute motor axonal neuropathy (AMAN) form of Guillain-Barré syndrome is characterized by sudden paralysis due to degeneration of nerve fibers and often preceded by infection by *Campylobacter jejuni* (a case-control study showed 26% vs. 2% in age-matched hospital controls), a Gram-negative bacteria in the gastrointestinal tract. The presence of the common moiety of antigenic oligosaccharides between the bacterial LPS and GM1 ganglioside in the nodes of Ranvier of myelinated nerve fibers and anti-GM1 antibody in patients strongly support the autoimmune nature of this disorder by the carbohydrate mimicry [1] (Fig. 4.1). In both the autopsy samples of fatal cases with median survival of 8 days and its GM1-immunized rabbit models, immunohistochemical analysis has revealed little

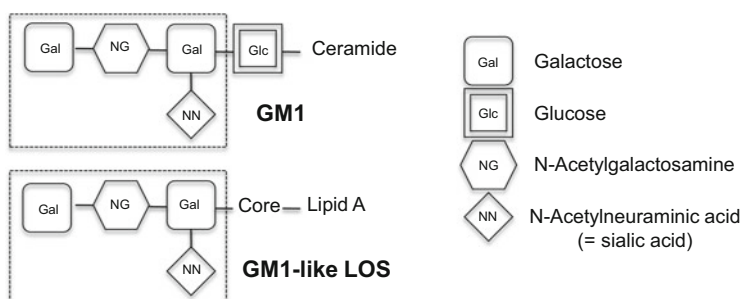


Fig. 4.1 Carbohydrate mimicry. Sugars are attached to ceramide (see Fig. 3.4 in Chap. 3) in GM1 in neurons. LPS (see Fig. 2.5 in Chap. 2) from a strain of *Campylobacter jejuni* from the AMAN patient is modified to GM1-like lipo-oligosaccharide (LOS) that resembles GM1 in the boxed area serving as an antigen for auto-immune responses

demyelination and lymphocytic infiltration but presence of macrophages scavenging the damaged axons and deposition of IgG and complement C3d in the nodal axolemma. The cluster of voltage-gated Na channel that is localized in the node of Ranvier disappears, resulting in failure of nerve conduction, which is followed by axonal degeneration at later stages.

4.2.3.2 Multiple Sclerosis

Multiple sclerosis (MS) is characterized by activation of autoreactive T cells specifically against the self-myelin that infiltrate the central nervous system, which is accompanied by demyelination of neurons, apoptosis, and edema. Vascular permeability, one of the essential features of inflammation, is increased in the blood-brain barrier (BBB) [2]. This enables the assessment of disease progression by brain magnetic resonance imaging (MRI) with gadolinium-DTPA. The immunological characteristics include DC expressing IL-12 and IL-23, Th1 secreting IFN γ , and Th17 producing IL-17 but lack Th2 expressing IL-4. Experimental allergic encephalitis (EAE), which is induced in susceptible animals by immunization with myelin proteins, provides a good animal model for MS, recapitulating the immune responses. IL-23 is required for T cells to make an entry into the central nervous system and is critical for Th17 polarization. As readers can see, MS has unbalanced coupling between DC and Th, favoring Th1 over Th2. Briefly, Th2 excess promotes allergy and Th1 overstimulation results in autoimmune disorders. Fumarate, which can induce IL-4-expressing Th2, is in clinical trials. The mechanisms involve the Nrf2-Keap1 system, one of the defense programs against ROS. In EAE model mice with CCR2-RFP and CX3CR1-GFP, the inflammatory lesion was found to be accumulated with monocyte-derived macrophages that mediated myelin destruction while microglia appeared to be engaged in disposal of the debris [3].

The brain of MS patients has infiltrating Th1 cells expressing IFN γ and Th17 secreting IL-17 and IL-22 through the BBB [4]. Individual stimulation by IL-17 and IL-22 of monolayers of human BBB-EC in Transwell or mouse brain endothelial cells immortalized by the polyoma middle T antigen (bEnd.3 cells) on electrodes induced enhanced permeability as judged by diffusion of fluorescent BSA or change of TER. The direct effects were accompanied by decreased expression of occludin and disorganized ZO-1, both of which have a major contribution to TJ. In addition, IL-17 induced Nox-2-dependent production of ROS, which activate phosphorylation of myosin light chain (pMLC). Expression of CCL2, IL-6, and IL-8 in IL-22-stimulated human BBB endothelial cells coincided with transendothelial migration of isolated CD4 $^{+}$ T cells in vitro [5]. IL-17 knockout mice under EAE had decreased ROS, pMLC, and extravasated IgG. The less severe but still progressive EAE in those mice simply means that IL-17 is just part of the pathogenesis.

During the preclinical period of EAE, both G-CSF and CXCL1 levels are increased in the serum with concomitant expansion of myeloid cells in the bone marrow. IL-17 mediates neutrophil mobilization to the brain during this period,

although neutrophils are not poor in number after EAE becomes mature. EAE after an adoptive transfer of myelin-reactive Th17 cells was abrogated in G-CSF receptor-KO mice treated by anti-CXCR2 blocking serum [6].

Src-dependent VE-cadherin tyrosine phosphorylation and associated disassembly of AJ was reported in CCL2. A destructive effect of IFN γ on EC is discussed in Chap. 11. Vascular permeability in the BBB is augmented as judged by gadolinium-MRI.

4.2.3.3 Pollinosis

It is not always the case that microbial invasion is an active process and attachment of pollen in respiratory mucosa is passive. Almost all pollens contain factors capable of producing ROS, and upon attachment on airway mucosa they are hydrolyzed to generate ROS to affect the epithelial cells [7]. Therefore, it is an active process by ROS. Within 15 min after attachment, ROS in the epithelial cells is detectable and eosinophilic infiltration is observed in 24–72 h. During the period in-between, chemical mediators are released upon pollen allergen binding to specific IgE attached to Fc ϵ RI on the surface of mast cells. The chemical mediators are stored in the granules of mast cells; therefore, the process is called degranulation. They are histamine, proteoglycans, such as heparin and chondroitin sulfate, and serine proteases, including chymase, PGD₂, LTB₄, LTC₄, and PAF. They cause vasodilatation and increased vasopermeability essential for pollen-induced inflammation. In the airways, contraction of smooth muscle takes place, potentially leading to asthmatic attack and respiratory failure. These are all early responses. Delayed reactions include leukocyte mobilization by released TNF α , CCL2, and IL-8.

The allergen stimulation induces Th2 with production of IL-4, IL-5, and IL-13, which promotes the class switch recombination in B cells to express IgE. T-bet-KO mice display Th2 dominance to induce bronchial asthma (see Chap. 2). Trials to shift the balance to Th1 over Th2 by CpG-activated TLR9 by vaccine in animal studies were done but in vain in terms of clinical practice. Here, we learn that the balance between Th1 and Th2 is tightly maintained in a homeostatic manner.

The allergens indicate those of nonmicrobial origin and capable of inducing IgE production. Most of them are proteins. They need to pass through the epithelial barrier to accomplish both Th2 induction and binding to specific IgE. Healthy barrier could defend perfectly, but a small injury could provide a chance for pollens to penetrate to induce inflammation.

For example, patients with loss of function mutations in filaggrin, which is a cytoskeletal molecule essential for skin barrier formation and hydration, suffer from ichthyosis vulgaris and atopic dermatitis [8]. Knock-in mice of filaggrin with an analogous mutation to human 5303delA exhibited dermatitis on percutaneous exposure of allergen ovalbumin. When the splenic cells from those mice were cultured *in vitro* with ovalbumin, Th cytokine responses were observed, including Th2 (IL-4, IL-5, and IL-13), Th1 (IFN γ), and Th17 (IL-17). Based on

the fact that Th1 through Th17 cytokines are involved in atopic dermatitis, completely humanized antibody against p40 (Ustekinumab), which is shared by receptors for IL-12 and IL-23, is in clinical trials.

As in the case of EAE just described, neutrophils are necessary for allergic contact dermatitis as shown by experiments with chemicals [9]. Neutrophils are recruited in the sensitization sites within 2–3 h. In both anti-Gr1 antibody-mediated depletion of neutrophils and myeloid-specific conditional deletion of Mcl-1 (see Chap. 14) in which mice are specifically deficient in neutrophils, sensitization and elicitation were inhibited. Mast cells appeared to mediate the process.

It is noteworthy that patients are compromised by bronchial asthma, although flaggrin is not expressed in airway epithelial cells. This indicates that the initial site of sensitization or triggering stimuli does not necessarily induce inflammation in the same site, and induction of inflammation in other sites of the body is one of the features of immunological inflammation. This is analogous to the idea of inflammation in premetastatic lungs, as described at the end of this book.

Both nasal and lacrimal discharges are caused by vasodilatation and vasopermeability, which are induced by histamine and LTC₄ [10]. Inhibitors against H1 receptor and CysLT₁ receptor are effective. The histamine receptor H1 is a 7-membrane spanning receptor couples with Gq/11 and activates phospholipase C. The product IP₃ and DAG induce Ca mobilization and PKC activation, respectively. The Ca²⁺-dependent pathway activates myosin light chain kinase (MLCK) in a Ca-calmodulin-dependent manner, ultimately leading to phosphorylation of MLC—an essential regulator of the actin-myosin interaction. MLCK in endothelial cells makes a complex with Src and cortactin that serves as a substrate of Src and an actin-binding protein. Vascular permeability is disrupted in MLCK-KO mice. A Ca²⁺ chelator BAPTA or a calmodulin inhibitor trifluoperazine can completely abolish the decrease of TER, indicating that the Ca²⁺ pathway plays a major role in histamine-induced vascular permeability.

On the other hand, IP₃ is recognized by and able to activate the IP₃ receptor in ER with Ca²⁺ channel function. After deprivation of Ca²⁺ in ER, a nonspecific cation channel TRPC1 (transient receptor potential channel) is activated in endothelial cells and Ca²⁺ is supplied from the extracellular space.

Cysteinyl-leukotrienes (CysLT), such as LTC₄ (Fig. 1.4 in Chap. 1), was shown to induce Ca²⁺ influx specifically through CysLT₂R and cell contraction in HUVEC in a ROCK-dependent manner. Notably, the CysLT₂R signaling potentiated the TNF α -induced HUVEC interaction with human monocyte cell line THP-1 by increasing the expression of VCAM-1. By contrast, CysLT₁R was specifically used in the LTC₄ stimulated HUVEC proliferation.

It may be difficult to see the acute effects of autoantibody experiment-wise. However, allergic inflammation caused by nonmicrobial antigens, such as pollens, also belongs to sterile inflammation. In this case, tissue mast cells and eosinophilic leukocytes play an essential role by releasing vasoactive substances, including histamine. Prominent nasal discharge results from the increased vascular permeability.

4.2.3.4 Psoriasis

Th17 cells are abundantly found in this chronic dermatitis lesion and play a significant role in promoting inflammation. Anti-TNF α antibody therapy results in a decrease of IL-23 and Th17 cells. Both anti-IL-17 (brodalumab) and IL-23 (against the IL-23-specific p19 subunit but not to the p40 subunit that is shared by IL-12) selective antibodies are currently undergoing clinical trials with good results.

4.3 X-ray Effects

X-ray at the dose of 20–40 Gy can induce sterile inflammation in the brain. The early study with rats showed that irradiation at 40 Gy caused active leakage of endogenous serum albumin. This was observed 1 day after irradiation, peaked at 3 days, and ameliorated at 30 days. A similar phenomenon was observed in monkey brain that was exposed to X-ray at 27 Gy. Electron microscopic examination revealed proteinous edema in the perivascular spaces with an increased number of pinocytotic vesicles in the endothelial cells, suggesting active absorption of extracellular fluid. This returned to the previous state 6 days after irradiation. Although precise mechanisms are not clear, X-ray-mediated changes in proteins and/or DNAs could alter the homeostasis of the BBB, resulting in increased vascular permeability reliably representative of inflammation.

Gamma knife has been utilized in many clinical settings, such as brain tumor destruction and curing trigeminal neuralgia [11]. It can target a highly focused site to avoid unnecessary damage of other tissues. In both rats and monkeys, macrophage infiltrations are observed 1–3 months postirradiation.

4.4 Analytical Research in Leukocyte Chemotaxis in Vivo

4.4.1 Real-Time Imaging

There are two modes of cell migration in general. The first depends on the gradient of a ligand to which a corresponding receptor on the surface of the cells binds, resulting in their locomotive action. Therefore, the cells migrate toward the ligand producers. The second mode is random cell migration with a particular direction to move, and the activity is intrinsic to the cells.

One of the most sophisticated ways to analytically observe the occurrence of sterile inflammation is the real-time imaging study after laser (Light Amplification by Stimulated Emission of Radiation) exposure and depends on the progress of the application of physical techniques to medicine. Its high spatial and temporal

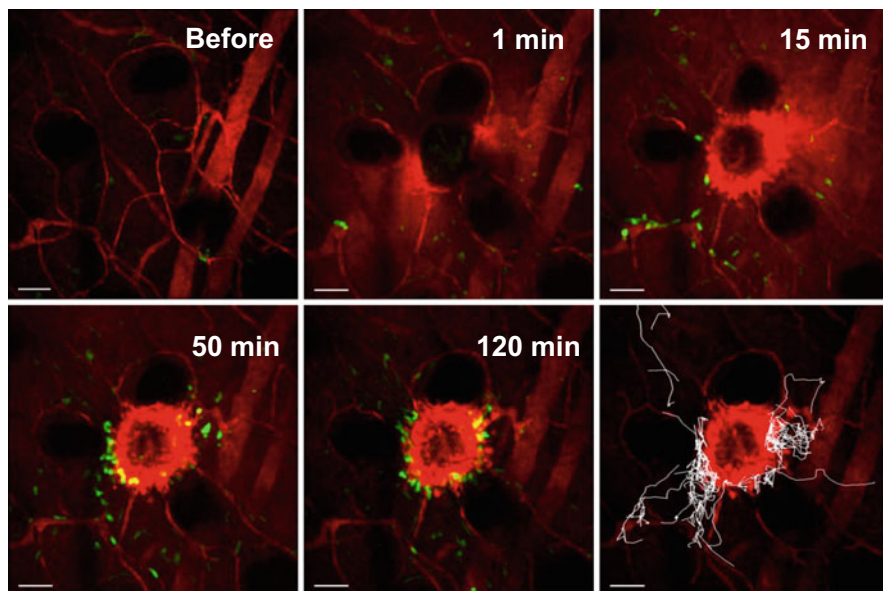


Fig. 4.2 Intravital leukocyte chemotaxis after laser exposure. See the text for detail. The time-lapse images are kindly provided by NG Laiguan, Singapore. Stack size: 60 μm , Step size: 4 μm

coherence enables it to be focused accurately to a given spot in tissues, expanding its application in medicine for laser surgery and so forth. An intense, multiphoton laser pulse of near far infrared beams (femtosecond pulsed, ~ 800 nm) induces tissue damages in a highly focused manner by thermal injury in a restricted area in the ear dermis.

As shown in Fig. 4.2, GFP-signaling neutrophils circulate, extravasate, and gather around the laser-exposed lesion [12]. The mice were engineered by knocking the enhanced GFP gene into the murine lysozyme locus (LysM-GFP mice). The GFP cells with high intensity were reported to be 52% mature neutrophil granulocytes, 40% myelocytes, and 4% monocytes in their bone marrow. Cells of weak intensity were monocytes and macrophages. Real-time intravital imaging examination revealed that leukocyte navigation consists of at least three phases. The scouting phase of ~ 15 min, the synchronized swarming phase of ~ 30 min, before the stabilization phase in which stable neutrophil clusters are observed for ~ 60 min. The leukocytes need to extravasate first, then migrate toward the damaged lesion, and finally form clusters. Studies that focused on the second swarming phase of interstitial migration could be achieved by transferring labeled leukocytes directly into the dermis before the laser ablation. The velocity of leukocytes in the interstitial migration is reported to be rather high around $10\text{--}20$ $\mu\text{m}/\text{min}$ in the perfect absence of blood flow, which is approximately one hundredth of the mean rheological speed in capillary.

Macrophage recruitment also is observed, but they stay at the peripheral regions of the leukocyte cluster. Although laser can destroy vessels with expected passive

leakage of plasma, which subsequently activates both platelet and coagulation system, recruitment of CD41-YFP platelet at the laser sites are not prominent (N.G. Laiguan, personal communication).

Then, what is the mechanism of chemotactic migration of leukocytes in the interstitial space? There is an elegant system by which to uncover the underlying molecular events. Injection of sorted and labeled leukocytes from recipient mice into dermal tissue of host mice and subsequent time-lapse imaging analysis allowed rather precise quantification of their chemotaxis. By utilizing gene-knockout mice in both recipient and host, leukotriene B4 (LTB4) (Fig. 1.4 in Chap. 1) has been shown to be a key molecule responsible for interstitial chemotaxis in the swarming phase [13]. Cell death of initially recruited leukocytes in the laser-damaged lesions could supply a small amount of leukocyte-derived LTB4, which triggers the amplification of LTB4-LTB4R1 auto- or paracrine signaling. Because cluster formation also is impaired in mice deficient in LTB4R1, the signaling is likely to confer an adhesive property to the recruiting leukocytes.

4.4.2 *Necessary and Sufficient Logics*

We discuss how to prove that molecule R plays a crucial role in a certain biological phenomenon. From a pharmacological point of view, activity of agonist A is the function of A bound to its receptor R, namely AR, but not unoccupied R or free A alone. A more complicated question is whether the reaction $A + R = AR$ is the critical mechanism by which to result in the biological consequence. This experimental situation is frequently encountered in scientific researchers to demonstrate all the facts and claims. All of the statements in the review book are ideally based on the logically designed experimental results. For this reason, it is worth stating in detail with the above-mentioned intravital leukocyte chemotaxis assay using intra-dermal injection procedure as a good example (Fig. 4.3).

Condition – In this experimental system, the intensity of interstitial chemotaxis is the biological outcome. The injected leukocytes are isolated from one mouse, labeled, and transferred to another mouse in which its own leukocytes are found in the injected area. R is fixed at the cell membrane, but A is derived from either injected leukocytes or from host cells and is free to move in the interstitial space.

Expression of R and A in both injected recipient and host cells can be controlled by utilizing knockout mice deficient in either R or A. We designate R+ or R– as cells wild-type or defective in R, respectively. When R+A+ was injected into R+A+ host, interstitial chemotaxis takes place as described earlier. If R–A+ was injected into R+A+ dermis, the chemotaxis was impaired, indicating that R is necessary. To prove that R is sufficient, R+A+ needs to rescue the chemotaxis-defective host background of R–A+.

If A is an authentic and specific ligand or agonist for R, one should show that A is necessary and sufficient to demonstrate the reaction: $A + R = AR$ is necessary and sufficient. How do we prove that A, which is free to move in the interstitial

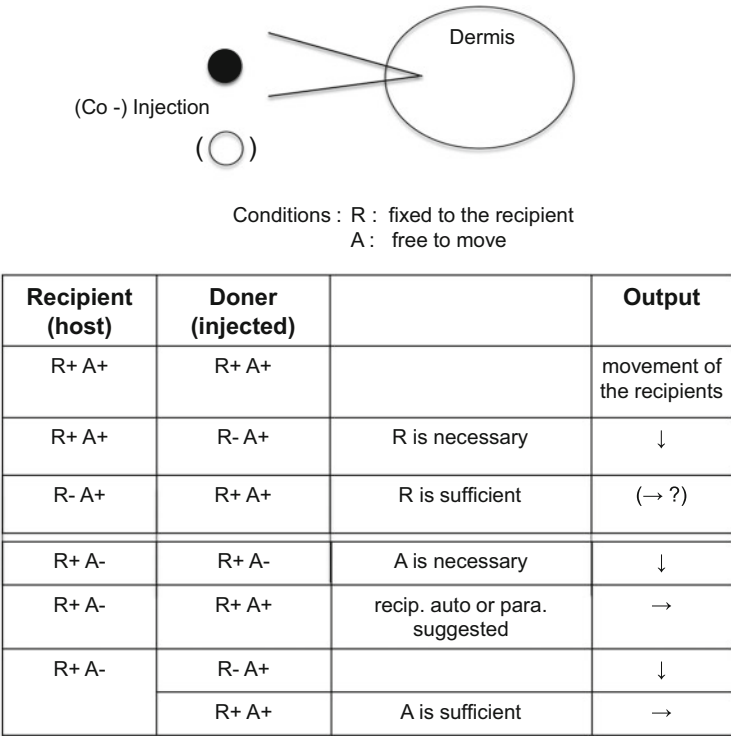


Fig. 4.3 Logics. See the text for detail

space, is necessary and sufficient for the chemotaxis? When R+A– is injected into R+A– dermis, the chemotaxis was inhibited, showing that A is necessary. R+A+ needs to rescue this defective condition. When R+A+ is injected into R+A– dermis, the chemotaxis was restored, indicating that A is sufficient, because R+A+ leukocytes are the only source of A in the injected dermis. The co-injected R-A+ with R +A+ was defective not only in R+A+ dermis but also in R+A– dermis, indicating that A is sufficient and binding of R to A is sufficient.

Tested R includes Fpr1, Fpr2, Cxcr2, C5ar1, Ccr1, Ccr2 Ccr5, Cxcr6, Ptafr, P2ry2, Il1r1, Tnfrsf1a, and P2rx7 in addition to the knockout mice deficient in signaling molecules, such as Myd88 and Gai2.

4.4.3 Monocyte-Dependent Neutrophil Extravasation in Lung Model

The aforementioned experimental system is rather watertight in a theoretical sense, and I do not intend to state that all the leukocyte migration to the inflamed lesion can be explained by that information [13]. For example, leukocytes need to migrate

transendothelially first, in the interstitial space, and if necessary, as in the case of standard pneumonia in which microbes are found in the air space, migrate transepithelially.

A substantial number of neutrophils are observed to stay as residents in lungs but not in other organs, such as brain and liver, in LysM-GFP mice. Intratracheal administration of bacteria *Listeria monocytogenes* or *Escherichia coli* BioParticles induced extravasation, interstitial migration, and cluster formation of neutrophils. This enhanced the neutrophil influx speed from 2.88 to 9.68 $\mu\text{m}/\text{min}$. Transplantation of wild-type lung to LysM-GFP mice as recipients gave similar neutrophil dynamics with GFP-monitored neutrophils being exclusively from LysM-GFP and extravasating from circulation. The transplantation causes ischemia reperfusion injury in the donor lung. GFP highly positive cells are Gr1⁺, Ly6G⁺, CD11b⁺, and CD115 (M-CSF receptor)⁺ neutrophils, and their clusters are associated with F4/80⁺, Ly6C⁺, and CD115⁺ monocytes, which also extravasate from circulation [14]. Clodronate liposome-mediated depletion of monocytes in recipient mice before lung transplantation abrogated the neutrophil extravasation.

Principle on which Clodronate Impairs Macrophages

Clodronate belongs to bisphosphonates clinically applied for the treatment of bone resorption disorders [15]. Its metabolite also is cytotoxic to macrophages by abrogating mitochondrial oxygen consumption competitively inhibiting ADP-ATP translocase. The liposome-mediated drug delivery confers cell specificity on the effect. When amphipathic phospholipid is dispersed in an aqueous solution, strongly hydrophilic clodronate is encapsulated by the artificially formed phospholipid bilayers, liposome. If their size is $>0.2 \mu\text{m}$, they are selectively phagocytosed by macrophages, which is followed by their disruption by lysosomal phospholipases to release clodronate in the cytoplasm. The drug is short-lived in circulation unless taken up by macrophages, which explains the resistance of nonphagocytic cells.

Intratracheal instillation of LPS causes alveolar accumulation of neutrophils [16]. This can be blocked by anti-CCR2 antibody or in the background of CCR2 knockout mice.

4.4.4 Focal Thermal Injury in Liver

In a murine model of focal thermal injury on the hepatic surface using LysM-GFP mice, neutrophils begin to adhere to the sinusoidal endothelial cells via Mac1-ICAM-1 interaction in 30 min (Fig. 1.2 in Chap. 1) [17]. The majority of neutrophils gather around the necrotic lesion through intravascular route rather than

transendothelial pathway. They reported no platelet thrombus in the nearby region of necrosis. In this system, application of an exogenous ATPase, apyrase, or suramin to inhibit P2Y purinergic receptor, or P2rx7 knockout background, decreased the total number of recruited neutrophils but failed to suppress their chemotactic behavior to the lesion. Administration of clodronate to deplete intrahepatic resident macrophage Kupffer cells had similar results. The ATP-P2X7R signaling up-regulates IL-1 β secretion, which then induces expression of ICAM-1 to promote neutrophil adherence.

4.4.5 CXCL2-CXCR2 System

Neutrophils in CXCR2-deficient mice showed no chemotactic intravascular migration to the damaged lesion along the concentration gradient of MIP-2 (= CXCL2) (Table 2.2 in Chap. 2) in the luminal side of endothelial cells [18]. Therefore, CXCL2-CXCR2 system appears to be necessary for the intravascular chemotaxis before the damaged lesion. However, chemotactic migration of neutrophils continues within the damaged and, therefore, necrotic area even though the CXCL2 concentration is the highest in the boundary area between damaged and nondamaged area and it drops down when getting close to the necrotic center. In this area, some substance(s) released from the necrotic tissue seems to activate formyl peptide receptor FPR1 to override CXCL2-dependent locomotive action. However, FPR1 knockout neutrophils lose directionality but retain the random movement velocity, suggesting that FPR1 is likely to govern which direction to migrate.

4.4.6 Sterile Inflammation Induced by Necrotic Cells

There is another system by which to study sterile inflammation caused by tissue injury. When cells are heat-shocked at 45 °C for 10 min or UV-irradiated, followed by incubation at 37 °C for 5 h, they become necrotic as judged by annexin V- and 7-aminoactinomycin D (AAD)-dually stained flow cytometry. Direct injection of those necrotic cells into peritoneal cavity of mice gives infiltrating neutrophils in the cavity 16 h after injection. Experiments conducted with a variety of knockout mice demonstrated that the phenomenon is dependent on IL-1R (Fig. 8.1 in Chap. 8) but not on TLR 1, 3, 6, 7, 9, 11, and IL-18R [19]. TLR2 and TLR4 knockout mice showed a partial response.

4.4.7 *Negative Chemotactism*

In a renal injury model in which TLR7-mediated immune activation is involved, Ly6C^{low} monocytes (see Ly6C subpopulations in Chap. 13) patrol endothelial cells of the kidney cortex in a manner dependent on binding between LFA1 and ICAM-1 (see Fig. 1.2 in Chap. 1), and in response to local TLR7 signal CX3CL1 (see Table 2.2 in Chap. 2) expression is up-regulated. This is followed by recruitment of neutrophils capable of causing necrosis of endothelial cells. As observed in this model, mobilized leukocytes do not necessarily extravasate, but instead may stay within the lumen. CX3CL1 appears to be required for the intravascular retention [20]. Mechanistically, this is mediated by inhibition of TGF β signaling in a manner dependent on Nr4a1 that belongs to the steroid/thyroid hormone receptor family [21].

4.5 Concluding Remarks

Sterile inflammation is caused by immune responses against nonmicrobial antigens, including those of host origin, and is closely associated with neutrophils chemotaxis to the injured spots. In organs, such as lung, where microbial assaults are constant, an interplay between monocytes and neutrophils takes place especially in transendothelial migration of leukocytes into the interstitial space. Degradation products of ECM and originally intracellular proteins released by necrotic cells into extracellular spaces serve as damage-associated molecular pattern (DAMP) to activate inflammatory responses. This will be discussed in detail in Part 2.

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Part II

What Is Endogenous Ligand

Chapter 5

Issue of Self and Non-self

5.1 Overview

The interactions between human and other species have been well documented in anthropology, because human beings are omnivorous. Cooking by boiling can fend off encounters with microbial toxins derived from their growth in foods. For human beings as a self, the most obvious non-self is other species, such as botany and reptiles. *Ophiophagus hannah* (king cobra) can kill humans by its neurotoxin acting on nicotinic acetylcholine receptor (nAChR) in humans [1]. In addition to biting, the ancient ways of transferring molecules to the circulation include inhalation of opium and the arrow poison used by South American Indians to hunt animals, such as curare containing d-tubocurarine that can block nAChR. However, it is needless to say that the most primitive route is per oral administration. Ingested and subsequently absorbed hazardous non-self substances are sensed by the chemoreceptor trigger zone in the midbrain and vomiting reaction takes place to expel the non-self from the self. The principle is that molecules from the non-self can be life-threatening for the self and the biological manifestation includes elimination of non-self or damage of self. The molecular basis is the interaction and subsequent events between a non-self-derived or exogenous molecule and a self-derived or endogenous receptor(s). The responsible sensors are expressed in immune cells, epithelial cells, and neuronal cells.

In Chap. 5, I will convince readers of the following issue. Irrespective of the difference in species, a substance derived from either non-self or self, occasionally accompanied by changes in quantity and/or quality, is non-self if recognized by self as life-threatening (Fig. 5.1). Both self and non-self substances induce inflammation by activating immune system either directly or indirectly through endogenous mediators.

		Endogenous receptor	
Bacteria			
▪ Gram-negative			
LPS	TLR4	—	danger
B12	Transcobalamin	—	nutrient
▪ Gram-positive			
Lipoteichoic acid	TLR2	—	danger
InlB(<i>Listeria monocytogenes</i> , GPR)	HGFR	—	?
InlA(<i>Listeria monocytogenes</i>)	E-cadherin	—	danger
Fungi			
▪ Tacrolimus	Calcineurin	—	?
▪ Rapamycin	mTOR	—	?
Virus			
▪ ssRNA (Influenza)	TLR7	—	danger
Plant			
▪ Digitalis (foxglove)	Na-K ATPase		
▪ Toxins			
▪ colchicine	β-tubulin	—	toxin
▪ paclitaxel	β-tubulin, TLR4	—	toxin
▪ vinca alkaloids	tubulin	—	toxin
			) danger
▪ Morphine	MOP(μ)	—	danger
▪ Cannabinoid	CB1 / CB2	—	danger
▪ Capsaicin	VRPV1	—	danger
Fish			
▪ TTX	Na-channel	—	toxin
Snake			
▪ Cobratoxin	nAchR	—	toxin

Fig. 5.1 Endogenous receptors for non-self ligands. All non-self ligands are recognized as a toxin or danger except for vitamin B12 serving as a nutrient. Fungi protect themselves against bacteria by producing bacteriocidal molecules as exemplified by penicillin. Rapamycin also is from fungi, and it is useful in some conditions in which its receptor mTOR is hyperactivated to contribute to the disease progression

5.2 Microbes

More easily understandable non-self is microbes. In order not to allow microbial (which includes viruses, bacteria, and fungi [see definition in Chap. 2]) invasion, the initial microbial assault causes their recognition by the host to defend against the second attack. The reactions to non-self are based on both innate and adaptive immunological systems and sensors or receptors for the non-self prepared before and after the exposure.

5.2.1 DNA and RNA

For example, in influenza virus infection, virus single strand RNAs (ssRNAs) are sensed by TLR7 with no specificity for sequence or virus at the beginning of infection and later-coming antibodies specifically against the influenza neuraminidase protein not only inhibit the expansion of the virus by neutralization but also serve as the receptor for the influenza virus. Viral or bacterial nonmethylated DNA is sensed by TLR9. Both TLR7 and TLR9 reside within ER. Double-stranded RNA viruses are sensed by TLR3 and RIG-I to induce IFN β . In TLR9 knockout mice, however, induction of type I interferon, such as IFN β , still takes place when cells are infected with *Listeria monocytogenes* known to transfer DNA during infection. Extracts prepared from *Listeria*, but not those treated by DNase, induced expression of both IL-6 and IFN β in a MyD88-TRIF-independent manner. This suggests that other DNA sensors exist to exert innate immune responses and they include DAI that binds dsDNAs to induce IFN β in a STING-modulated manner, and AIM2 that binds both bacterial and viral dsDNAs to activate NLRP3 inflammasome to produce IL-1 β [2, 3]. It is noteworthy that triple gene knockout of HMGB1, HMGB2 and HMGB3 resulted in failure of AIM2-dependent IL-1 β production. TLR7 or TLR9-mediated expression of type 1 IFN are impaired in HMGB1-deficient cells. The information suggests more promiscuous role of HMGB in foreign DNA sensing programs [4].

5.2.2 Lipopolysaccharide

Structure of lipopolysaccharide (LPS) is depicted in Fig. 2.5 in Chap. 2.

5.2.2.1 LBP-CD14-TLR4/MD-2 Cascade (First Biosensor)

The endotoxin or LPS produced from Gram-negative bacteria, which is non-self, can bind the host TLR4-MD-2 complex, which is self, to exert immune responses. For LPS to be recognized by the TLR4-MD-2 complex, LPS needs to be conveyed

by several transporters that also can recognize LPS but without signaling to exert immune responses (Fig. 5.2). LPS released into the serum from bacterial cell wall forms a micelle structure, an aggregate with hydrophilic portions facing the surrounding serum and hydrophobic regions of acyl residues of lipid A lined toward the micelle center. The lipid transfer glycoprotein called LPS-binding protein (LBP), which has positive charges, binds the negatively charged LPS and participates in the catalytic transfer of monomeric LPS from the micelle to another glycoprotein CD14 [5–8]. For example, a single molecule LBP can facilitate transfer of more than 100 molecules of LPS to CD14 in 30 min. LBP is an acute phase reactant produced in the liver, whose serum levels are 200–500 ng/ml in

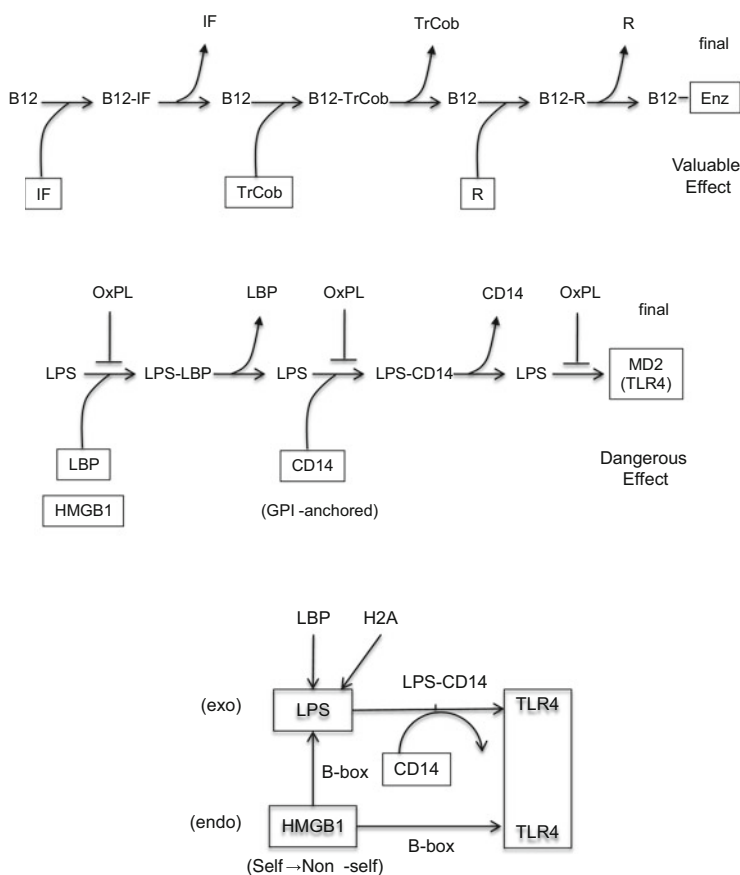


Fig. 5.2 Receptor-mediated sequential transfer of non-self ligands. The essential nutrient B12 as a non-self is sequentially transferred by gastric intrinsic factor (*IF*), serum transcobalamin (*TrCob*), and finally passed onto receptor for endocytosis in cells, and then used as a cofactor for metabolic enzymes (*Enz*). The danger non-self LPS is transferred to the final sensor, the TLR4-MD-2 complex by a series of endogenous receptors 1. Each transfer is blocked by OxPL (see Chap. 6). HMGB1 participates in the sensing of DNAs but also of LPS by even directly binding LPS

physiological conditions and increase to 50 $\mu\text{g/ml}$ after an induction of acute phase response. CD14 has both glycosylphosphatidyl inositol (GPI)-anchored, membrane-bound (designated as just CD14), and soluble forms (sCD14). Sequential binding of LPS to LBP followed by CD14 is eventually and efficiently passed onto the TLR4-MD-2 complex in cells like macrophages. In cells devoid of CD14 as observed in endothelial cells and epithelial cells, sCD14 substitutes for CD14 by conveying LPS to the TLR4/MD-2 complex.

The dissociation constant (K_d) of LPS from *Salmonella minnesota* Re595 versus purified LBP from rabbit serum was documented to be 3.5 nM and that of the same LPS versus soluble form of sCD14 purified from recombinant CHO cells was 29 nM [9]. Scatchard analysis with [^3H]-lipid A and Ba/F3 cells stably expressing the mouse TLR4/MD-2 complex gave K_d of 3–10 nM [8, 10]. Other binding studies with fluorescent FITC-labeled LPS and baculovirus-purified MD-2 proteins provided K_d of 65 nM [11].

Dissociation Constant (K_d)

The principle of equilibrium should be remembered. $A + B = AB$ is expressed with k_{on} (direction to AB) and k_{off} constants. When equilibrium is reached $A \times B \times k_{on} = AB \times k_{off}$. The dissociation constant K_d is defined $k_{off}/k_{on} = A \times B/AB$. Therefore, the smaller the K_d , the stronger the binding between A and B. The binding or association constant is defined as $K_a = k_{on}/k_{off}$. A group reported a K_a of $1.65 \times 10^4 \text{ (g/ml)}^{-1}$ by using capillary zone electrophoresis with commercially available LPS (Sigma) and TLR4 (R&D) [12]. Suppose that MW of LPS is 100 kDa; K_d is roughly 600 nM.

CD14-dependent endocytosis of TLR4 is necessary for TRIF-mediated $\text{IFN}\beta$ production [13]. In CD14-KO mouse bone marrow macrophages, both high (0.1–1.0 $\mu\text{g/ml}$) and low (approximately 0.01 $\mu\text{g/ml}$ = 10 ng/ml) dosages of LPS failed to induce $\text{IFN}\beta$ production. However, it also was found that while the high dosages of LPS induced $\text{TNF}\alpha$ production and release in a CD14-independent manner, CD14 was found to be necessary for the low dosages of LPS to cause $\text{TNF}\alpha$ expression. The aforementioned criteria of high versus low dosages of LPS do not apply to what is described in the next chapter (10 ng/ml is still high in the discussion described next). Thus, the efficient transfer of LPS to the TLR4/MD-2 complex and/or their endocytosis is dependent on CD14.

No significant differences were found in the serum $\text{TNF}\alpha$ levels between wild-type and LBP-knockout mice, both of which were injected with 0.02–2.0 μg of LPS per mouse. This implicates the presence of other vehicle proteins in circulation than LBP by which to transfer LPS to the subsequent binding proteins. One candidate is HMGB1 (see details for the original characteristics of HMGB1 in Chap. 6).

HMGB1 was documented to be under detection in the normal human serum (<50 pg/ml), but the levels increase to 25.2 ng/ml and 83.7 ng/ml in survivors and nonsurvivors, respectively, of septic patients [14]. In experimental mice

intraperitoneally injected with LPS at 10 mg/kg, the serum HMGB1 levels started to increase at 8 h and peaked at 15 h; the plateau levels were maintained up to 32 h. This contrasts with the serum levels of early mediators of LPS, such as IL-1 β and TNF α that are released within 5 min. It is assumed that HMGB1 chaperones LPS in late phases, because it was reported that LBP competes with HMGB1 for LPS binding.

I summarize the LPS-binding proteins: LBP, HMGB1, CD14, and the TLR4-MD-2 complex. All of them are self and their cooperation allows the LPS-administered host to recognize the presence of non-self within self.

Sepsis is frequently lethal. This again provides a good biological system in which to assess what is essential to cause lethality with the necessary and sufficient logics. With knockout mice studies, it was shown that TLR4 but neither LBP nor TNF α is necessary for lethality, although there is a reciprocal activation of induction between the two cytokines (IL-1 β and TNF α) and HMGB1. Because anti-HMGB1 neutralizing antibody can rescue the lethality and HMGB1 injection alone causes lethality, it safely can be said that LPS (non-self) and HMGB1 (self) are necessary and sufficient for the lethality. HMGB1 is likely a reliable endogenous mediator. However, we will see a big issue in the following chapter where we discuss endogenous TLR4 ligands and HMGB1 is one of them.

5.2.2.2 Mutations in TLR4

Defective response to LPS, thus enhancing the susceptibility to Gram-negative bacteria, was reported in 1968 in C3H/HeJ mouse strain with the allele LPSd compared with control wild-type strain C3H/HeN with the LPSn allele and in C57BL/10ScCr strain versus control C57BL/10ScSn in 1978 [15]. Positional cloning in 1998 revealed that homozygous LPSd/LPSd caused the LPS unresponsiveness, and a single nucleotide change in the TLR4 gene resulted in a single amino acid substitution at 712 from Pro to His in the cytoplasmic TIR (TOLL/IL1R homology) domain.

Three exons of TLR4 were deleted in C57BL/10ScCr (null allele), resulting in no expression of TLR4 with a recessive trait. Mating studies showed that LPSn/null and LPSn/LPSn were equally responsive and null/null, and LPSd/LPSd were unresponsive, but LPSd/LPSn displayed an intermediate response to LPS. Therefore, LPSd is a codominant (a single gene has more than one dominant allele as exemplified in ABO blood group of AB in which A and B are codominant to each other) but working in a dominant negative fashion to LPSn. The Pro712His was found to abolish binding of signaling molecule MyD88 to the TIR domain (Fig. 5.3).

In humans, common polymorphism D299G and T399I was reported to be associated with reduced responses to LPS and preposition to severe malaria by *Plasmodium falciparum* [16]. Other mutations closely linked to meningitis by *Neisseria meningitides*, a Gram-negative diplococcus also releasing LPS upon infection, have been documented [17].

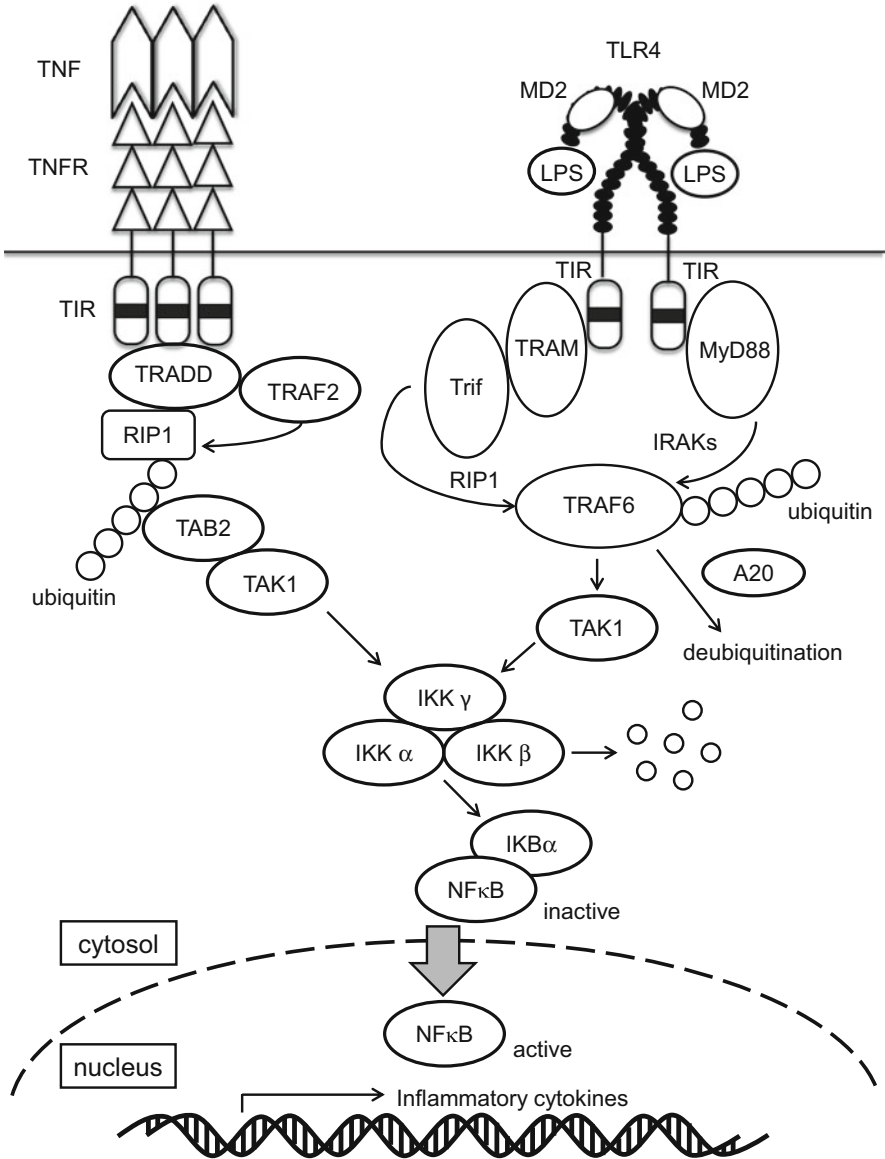


Fig. 5.3 LPS and TNF α signaling cascades. Adaptor molecules and kinases in regulation of signaling proteins by phosphorylation and protein degradation are shown. The signaling eventually results in NF κ B activation

5.2.2.3 BPI (Second Biosensor)

Different from the transporter-mediated signaling cascade (LBP-CD14-TLR4/MD-2), one more biosensor called bactericidal/permeability-increasing protein (BPI)

directly neutralize LPS in mucosal surfaces, such as intestinal and genital tracts, where homeostasis between host and microbe needs to be maintained [18, 19]. BPI is an antimicrobial peptide alternatively named as cationic antibacterial protein of 57 kD (CAP57), also expressed in myeloid cells and competes with LBP by binding to lipid A with a high affinity. A K_d 4.1 nM was reported in the binding assay with ^{125}I -labeled BPI and *Escherichia coli*-derived lipid A.

The outermost O antigen (see Fig. 2.5 in Chap. 2) is immunogenic, but due to its variable nature antibodies against it can hardly be of practical use in clinical settings. The lipid A portion of LPS is the most potent in biological activity, and lipid A by itself can be immunogenic but practically not since its antigenic determinants are buried in sugar residues and other surface components. Clinical trials to apply anti-lipid A antibodies to ameliorate septic shock has been unsuccessful. The cytokine production was not abrogated by those antibodies.

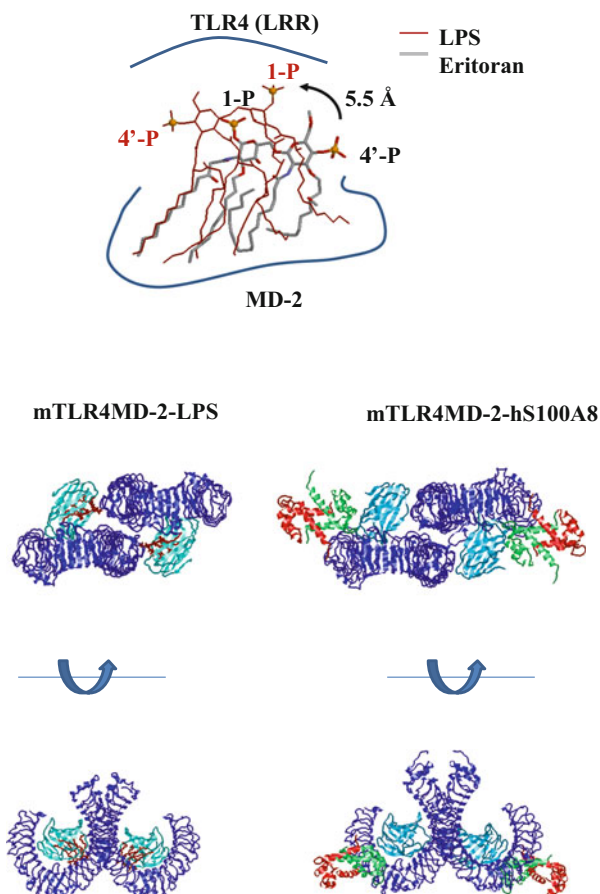
Lipid A, as mentioned earlier, is amphipathic and forms aggregates when its concentration is above a certain point called critical micellar (aggregate) concentration (CMC), which varies by orders of magnitude depending on the species of lipid A under different conditions but roughly 10^{-6} to 10^{-8} M. Structural prediction of aggregates has been made by physicochemical analysis of ratio of areas of hydrophilic polar relative to hydrophobic nonpolar region. If the ratio is less than 1, lipid A forms a lamellar structure being inactive to stimulate cytokine production. If it is greater than 1, it is in a cubic structure lined by its hydrated head. Monophosphoryl lipid A has mixed lamellar/cubic structures and intermediate activities.

5.2.2.4 TLR Family Constitutes 13 Mammalian Members

TLR4 was discovered in 1997 as a *Drosophila* Toll ortholog that is homologous over the whole protein sequence [20, 21]. One of the unique features of this family member is the presence of the leucine-rich repeat (LRR) domain consisting of 16–28 LRR modules [22]. In TLR1, TLR2, and TLR4, which have been co-crystallized with their respective ligand, the LRR module is 24 amino acids (xLxxLxxLxLxxNxLxxLPxxxFx). The LRR domain exhibits a unique horseshoe-like shape with the inner concave region contributed by the conserved sequence pattern LxxLxLxxN of the LRR module and with the convex region formed by the variable parts of the LRR module.

Lipoproteins are immunostimulatory membrane components of bacterial wall. A co-crystal structure of the heteroduplex of TLR1 and TLR2 bound to a synthetic derivative Pam3CSK4 of triacylated lipoprotein revealed that two lipid chains and the remaining one chain bind TLR2 and TLR1 at respective binding sites formed by the LRR domains. Binding between TLR4 and its coreceptor, MD-2, is achieved mainly by ionic and hydrogen bonds [23]. The core lipid A of LPS usually is hexa-acylated. Five of them bind the hydrophobic pocket of MD-2 and remaining one acyl chain binds the hydrophobic residues in TLR4, which is essential for heterodimerization. One of the antagonistic ligands eritoran binds the pocket of MD-2 but

Fig. 5.4 Structure of TLR4, LPS, and Eritoran. Crystal structure analysis revealed MD-2-mediated dimerization of TLR4. For short, TLR4 is recruited with MD-2 by 1:1 and two molecules of LPS bind the two TLR4-MD-2 complexes. By naming each as LPS-1 and -2, etc., LPS-1 binds the LRR of TLR4-1 and MD-2-2 while LPS-2 binds the LRR of TLR4-2 and MD-2-1. The interconnection induces dimerization and subsequent activation of the receptor complex. Docking simulation of an endogenous ligand S100A8 homo-dimer (see Chap. 15) and the TLR4-MD-2 complex also is provided



never interacts with TLR4 itself [24] (Fig. 5.4). In both TLR1-TLR2 and dimerized TLR4-MD-2, an “m”-shaped configuration of the dimerized receptor complex is supposed to be established, which in turn induces a proper orientation of the intracellular TIR domain followed by the recruitment of signaling molecules. The C-terminal LRR module of CD14 interacts with the same structure of another CD14, thereby forming a dimer with 22 LRR modules whose length is comparable to those of TLRs. The pockets in the N-terminal regions are the binding sites for LPS.

5.2.2.5 Caspase-11 (Third Biosensor)

In addition to the biosensor TLR4/MD-2 complex recognizing LPS, caspase-11 in the NLRP3 inflammasome (see Chap. 8 for details) has been shown to be activated by intracellular LPS even in the absence of TLR4, MD-2, and CD14 [25]. Their

homologs, such as RP105 (TLR4 homolog) and lipid A-binding protein MD-1, also are dispensable to activate the inflammasome to release IL-1 β and IL-18 in mouse bone marrow-derived macrophages. This indicates that LPS serves as an apparent agonist for the third biosensor caspase-11, which induces pyroptosis. Priming to up-regulated IL-1 β mRNA transcription by other signals like poly (I:C) also sensitizes the macrophages for LPS-induced lethality at small dosages.

The prototypic structure of LPS from *E. coli* has hexa-acyl and diphosphoryl lipid A. Caspase-11 is not activated by tetra-acylated lipid A from *Francisella novicida*, which confers an evasion strategy on this microbe.

5.2.2.6 RAGE (Fourth Biosensor)

The fourth biosensor for LPS or lipid A is RAGE (receptor for AGE) with K_d 35 nM and 2 nM, respectively, as judged by surface plasmon resonance analysis with purified human endogenous secretory RAGE and *E. coli* LPS [26]. In RAGE knockout mice, intraperitoneal injection of LPS resulted in reduced lethality as well as decreased serum levels of HMGB1 that also can bind RAGE.

5.2.2.7 Histones (Possible Fifth Biosensor)

During the research to look for amphiphilic proteins, such as surfactant protein-C in the preparation of mouse type II pneumocytes, reverse-phase HPLC and matrix-assisted laser desorption and ionization-time-of-flight (MALDI-TOF) techniques uncovered H2 and H4 histones as LPS-binding proteins [27, 28]. Under the condition where polymyxin B bound 3H-LPS derived from *S. minnesota* with K_d 1.1 μ M, all histones (H1, H2A, H2B, H3, and H4) bound the LPS with K_d ranging from 0.14 (H3) to 5.3 (H4) μ M.

Histones are usually localized in the nucleus but released from dying cells during injury and sepsis. Given the ability of a mixture of histones to activate platelet aggregation in a TLR4-dependent manner, the influence by LPS provided by Gram-negative bacteria is assumed on coagulation. In fact, histone H3 and H4 serve as endogenous mediators and therefore indirect sensors during sepsis, neutralizing antibody against which inhibited the lethality caused by their endothelial cytotoxicity.

5.2.3 Ancient to Modern Perspective

5.2.3.1 Limulus

Here is the chance to view host strategies of protection against non-self from an evolutionary perspective. The currently most sensitive test to prove the presence of

LPS is Limulus test [29, 30]. Amebocytes in the Atlantic (*Limulus polyphemus*) and Japanese (*Tachyleustridentatus*) horseshoe crabs, a marine arthropod found in the Gulf of Mexico and Seto Inland Sea, respectively, are highly sensitive to LPS of Gram-negative bacteria. The membrane-bound receptor Factor C upon association with LPS with a high affinity autocatalytically becomes activated as a serine protease and executes a hemolymph coagulation cascade by releasing clotting factor, including Factor B and G, by exocytosis and engulfs the invading microbes. Moreover, bacteria-killing substances, such as tachyplesins, are released from intracellular granules. The LPS-induced signaling involves trimeric GTP-binding proteins. Because the response is an enzymatic reaction of high stoichiometry, amplification of the readout gives a high sensitivity when utilized as a detection system for LPS (see Chap. 7 for demonstration of endogenous ligand for Limulus amebocyte lysate [LAL] test). This ancient biosensor (RWR as a tri-peptide) shares a conserved tri-peptide motif of an aromatic residue flanked by two basic residues with modern ones, including human LBP (RWK), MD-2 (KYK), and BPI (KWK). The LPS-binding ability was lost in Factor C with a RWR to EWE mutation. Serine protease zymogen system also is utilized in the modern clotting system in humans and in Snake and Easter located upstream of the *Drosophila* Spatzel-Toll system.

5.2.3.2 *Drosophila melanogaster*

In *Drosophila*, whose experimental system in biology has made a great contribution to the modern medical science, LPS fails to stimulate their immune system, in which hemocytes and the fat body equivalent to human white blood cells and liver, respectively, are responsive tissues. Despite their unresponsiveness to LPS, it is worth noting because of its mechanistic analogy of defense against assaults by non-self [31]. *Drosophila* senses peptidoglycan (PGN) that is the major cell wall component of both Gram-positive and -negative bacteria. Structurally, PGN is a polymer of Glc-Nac and Mur-Nac connected by a short stretch of amino acids. The sensor is called PGN-binding protein (PGRP) that comprises roughly 100 members. Like the antimicrobial peptide BPI in human, some PGRPs have amidase activity to destroy PGN to give detrimental influences on invading bacteria. Others are involved in the activation of phenoloxidase responsible for local wound healing, which is accompanied by the production of ROS and melanin toxic to microbes.

There are two pathways against PGN, i.e., the imd (immune deficiency) and Toll pathways [32]. The Toll gene was originally discovered in 1988 in *Drosophila melanogaster* as one of the genes responsible for formation of dorsoventral polarity in the early embryogenesis. Toll means <great> in German (Das ist eine tolle Idee = That is a great idea). In 1996, the Jule A. Hoffman group demonstrated that Toll controls antifungal response. A human homolog of *Drosophila* Toll was identified in 1997 by BLAST search and subsequent PCR cloning and named as Toll-like receptor 4 (TLR4) (see above).

The imd pathway is similar to the mammalian TNF α pathway, and in response to Gram-negative and positive bacteria antimicrobial peptides, such as Diptericin, are

produced. The Toll pathway is for Gram-positive bacteria and involved in the induction of peptides, including Drosomycin. By screening drugs capable of inhibiting the imd pathway, we have identified celastarmycin A, which was found to bind mTOC (mammalian target of celastarmycin A) and block TNF α -stimulated IL-8 production in mammalian cells [33]. A sensor of PGRP in the Toll pathway is PGRP-SA.

PGRP-SA, which by itself lacks amidase activity, serine protease activity, or phenoloxidase activity, initiates a serine protease cascade in which pro-Spatzle is cleaved to become Spatzle, the activated ligand for Toll receptor that provokes innate immune signaling. Therefore, instead of transferring LPS as non-self by LBP and sCD14 of endogenous origin to the final destination TLR4/MD-2 complex on the cell surface, PGN as non-self directly activates endogenous ligand for the membrane-localized receptor.

5.2.3.3 *Caenorhabditis elegans*

Although *C. elegans* has only one TLR homolog TOL-1 in their genome, it does not appear to play a role in resistance to pathogens by controlling immune effector molecules or cells [34, 35]. However, TOL-1 mutants were killed by *Salmonella enterica* and associated with poor expression of ABF-2, a defensin-like protein, in the pharynx.

5.2.4 Vitamin B12

Neither human nor plant can generate the well-known vitamin B12 [36]. It is synthesized in bacteria and stored in the liver of grass-feeding animals. Because vitamin B12 that is actually synthesized in our microbes in the colon needs the intrinsic factor secreted from stomach, which is located upstream of colon, for absorption in the ileum, we are eventually supplied with B12 from the outside environment. Valuable molecules, such as B12, are called nutrients, which interact with endogenous host molecules to maintain life. While bacteria-derived LPS is a toxin alarming the defense program, such as the immune system, bacteria-derived vitamin B12 is a so-called nutrient with no alarm and for valuable effects. What discriminates between those two non-self molecules of bacterial origin? An endogenous receptor in host cells is responsible for making the distinction.

From absorption to trafficking to proper compartments in suitable types of cells, B12 is carried by transporter proteins to the two essential final destinations: enzymatic alkylation reactions by methionine synthase and methylmalonyl-CoA mutase. In the former catalysis, for example, B12 serves as a cofactor, not cosubstrate that changes its structure after reaction, to produce tetrahydrofolate for the DNA synthesis. Therefore, there is more than one endogenous receptor that sequentially passes B12 to the next binding protein. The cell surface receptor for

B12 is a receptor for the carrier in blood called transcobalamin. Subsequent endocytosis is its major function, and the receptor does not transmit intracellular signaling. Conversely, deficiency of the nutrients results in disturbance of tissues in which the final products play an essential role. In the case of B12, poor synthesis of the product, DNA, causes the well-known pernicious anemia and accumulation of the substrate methyl-malonyl-CoA, which serves as an inhibitor for malonyl-CoA required for fatty acid synthesis, results in subacute combined degeneration, a neuronal disorder caused by the destruction of myelin sheath, a modified version of plasma membrane.

Collectively, there are at least two types of non-self of bacterial origin; noninvited and invited non-selfs. Both the presence of the noninvited and absence of the invited give an alert to the self to ameliorate the homeostatic disturbances.

5.3 Plant

Chinese herbal medicine has been established since a few thousand years ago without exactly identifying any single efficient molecule in the plant. In Japan, its modified version is currently put into clinical practice and is called Kampo: Japanese traditional medicine. In both, original Chinese or Japanese, any single drug, sho-saiko-to for example known for the treatment of chronic viral hepatitis, is a mixture of several plant extracts. Namely, they are opposite of Western medicine in which one substance by high-grade purification steps is usually on discussion for its efficacy. However, some of the effective drugs of current usage are derived from plant and chemical modification of the original substances under active experiment to obtain good drugs.

5.3.1 Microtubule-Binding Proteins

Taxane, vinca alkaloid, and colchicine are clinically well-known drugs of plant origin. Three of them bind microtubules that consist of heterodimers of α - and β -tubulin in a controlled equilibrium between polymerization and depolymerization.

5.3.1.1 Colchicine

The most classical one, colchicine, is produced in poisonous saffron *Colchicum autumnale* and copolymerized into microtubules by binding β -tubulin [37]. The binding distorts the microtubule structure and thereby inhibits polymerization. Since the polymerized microtubules are destabilized, colchicine is called a destabilizing binder. Vinblastine, one of the vinca alkaloids, also destabilizes

polymerization by binding the interface between α - and β -tubulin heterodimers but without copolymerization into microtubules. Taxanes, on the other hand, bind β -tubulins in the polymerized microtubules. The stabilized microtubules by taxanes lose their dynamics. Whether destabilizing or stabilizing, those binders can kill the cells by disrupting microtubules. Thus, in the case of microtubules as an endogenous receptor (self), there exist at least three corresponding non-selfs of plant origin. Colchicine also has been utilized as a useful drug for gout and familial Mediterranean fever (FMF) (see Chap. 8). Since deterioration of microtubules can block cell growth and cell migration, chemically modified versions of the natural stabilizers and destabilizers are under investigation for cancer therapeutic agents.

5.3.1.2 Paclitaxel

One of the taxanes, paclitaxel produced from the Pacific yew tree, not only causes tumor necrosis as an antineoplastic drug, which in turn activates the immune system via endogenous mediators, but also has been claimed to bind MD-2 directly and causes activation of NF κ B [38]. The minimum paclitaxel dosages are roughly 5 and 3 μ M for NF κ B activation and TNF α secretion in macrophages. The paclitaxel-induced NF κ B translocation to the nucleus was failed to be observed in macrophages from C3H/HeJ mice with mutationally defective TLR4. Thus, this non-self not only arrests cell cycle by binding to intracellular microtubules but also activates immune cells through the TLR4/MD-2 complex.

5.3.2 Morphine

Colchicine, paclitaxel, and morphine are apparently non-self and can cause harmful effects on the host cells. They are not necessary to maintain our usual life but still useful in medicine. The potent opiate morphine is derived from the plant the *Papaver somniferum* poppy and therefore clearly non-self. It binds endogenous receptor called MOP (μ) that plays an inhibitory role in the sensory nervous system for pain. In addition to the valuable analgesic effect, morphine when applied over a certain dosage can induce not only vomiting reflex by stimulating the chemoreceptor trigger zone located in the floor of the fourth ventricle but also respiratory arrest by inactivating the respiratory center in the brainstem.

Curcumin, one of the natural polyphenols found in the spice called turmeric, binds MD-2 with K_d 0.55 μ M. This binding is blocked by morphine with K_i 4.3 μ M. LPS also competes with morphine for MD-2 binding [39].

5.3.3 Neuronal Sensor for Non-self

Noxious temperature, irritating acid with low pH, and mechanical pressure are not clear non-self ligands, but they are certainly defended to avoid injury in the self. Capsaicin is an ingredient in chili peppers produced by a plant genus *Capsicum*. It causes burning sensation through binding to the intracellular region of transient receptor potential V1 (TRPV1), previously called vanilloid receptor 1, because the vanillyl group is functionally critical.). TRPV1 is a cation channel responsible for nociception and thermosensing, preferentially allowing nonselective inward currents of $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Na}^{+}$ [40–42]. Another agonist of natural occurrence resiniferatoxin from plant *Euphorbia resinifera* was discovered to have three to four orders of magnitude higher potency in the quantified by vascular permeability assay with Evans blue that is extravasated in the rat hind paw topically applied with the irritants (neurogenic inflammation) and levels of fall in rectal temperature after their subcutaneous injection (thermoregulation). Capsaicin and resiniferatoxin share the pharmacophoric vanilloid region but have different lipophilic structures. The receptor TRPV1 is present in afferent unmyelinated C fibers of the peripheral nervous system transmitting signals of pain and thermal and chemical stimuli. It is called nociceptive (neural processes of encoding and processing noxious stimuli) neurons. Interestingly, TRPV1 has been shown to be activated by heat up to 45 °C and low pH down to pH 5.5, indicating that those stimuli are sufficient to activate TRPV1 in vitro. In binding experiments with [³H]-labeled resiniferatoxin in HEK293 cells transfected with TRPV1, TRPV1 gave a K_d of 84 pM, which was inhibited by capsaicin with K_i value 4.0 μM . Without crystal structure, it is assumed that natural ligand binding and thermochemical effects might lead to the activated state of the receptor. However, the receptor also was demonstrated to be necessary for the in vivo responses to vanilloid substances, protons, and heat-mediated noxious stimuli by utilizing TRPV1 knockout mice. The finding that CCR2 is activated in peripheral nerve injury in which CCL2 is released and sensitize nociceptors suggests collaboration between immune and neuronal systems to recognize and cope with non-self matters [43].

5.4 PAR

Recognition of plant-derived non-self also is executed by the proteinase-activated receptor (PAR) system, which was described in Chap. 3 where the unique mode of its activation is discussed in detail. Different from PAR1, 3 and 4, PAR2 has a broader spectrum of activators, including not only endogenous ones, such as coagulation factor Xa, the soluble form β tryptase released from activated mast cells, and leukocyte cathepsin-G, but also exogenous ones. The bacterial protease gingipains-R and the house dust mite allergen Der P1 are included in the exogenous non-self protease [44]. Gingipains-R is a cysteine protease that is produced by

Porphyromonas gingivalis, a major microbe responsible for chronic periodontitis and activates PAR2 in oral epithelial cells. The cysteine protease Der P1 produced by *Dermatophagoides pteronyssinus* has been shown to increase epithelial permeability by degradation of tight junction proteins, such as ZO-1, thereby allowing or facilitating entry of inhaled allergens into subepithelial space where DCs are located. The receptor-coupled hetero-trimeric G protein-mediated signaling cascade eventually induces expression of PGE2 as well as proinflammatory cytokines, such as IL-6, in airway epithelial cells and vasodilatation that is at least partly mediated by NO release.

Recently, it was described that both of the non-self sensors TLR4 and PAR2 cooperates for influenza virus [45]. When TLR4-knockout macrophages were treated with PAR2 agonists of synthetic peptide, NF κ B-dependent expression of IL-1 β was significantly reduced compared with wild-type cells, suggesting that TLR4 is required for full activation of PAR2. Individual knockout mice of PAR2 and TLR4 are resistant to mouse-adapted H1N1 influenza virus-induced lethality.

5.5 Isoantigen as an Exogenous Ligand

It was not until the discovery of the ABO blood type by Landsteiner in 1901 that human beings obtained physical evidence to discriminate self from non-self in addition to sexual difference. The necessity of transferring non-self substances to a self by medical procedures, such as transplantation of blood cells, incited researchers because of the disastrous outcomes, such as mismatched transfusion (Fig. 5.5). I will briefly describe this isoantigen (also called alloantigen) of evolutionary importance in humans.

The isoantigens exist in subgroups of a species but not others and are represented by blood group and histocompatibility antigens. There are three genes: A, B, and O blood types. The A and B gene encodes α 1, 3 N-acetylgalactosaminyl (GalNAc) transferase and α 1, 3 galactosyltransferase, respectively [46]. Both glycosyltransferases differ in four consistent substitutions of nucleotides that change four amino acids, which results in substrate specificity of the enzymes UDP-GalNAc versus UDP-Gal. The O gene has a single nucleotide deletion in the coding region of the A gene leading to the generation of an inactive enzyme by a frame shift. Both of the polymorphic A and B genes are inherited on a Mendelian dominant pattern. The sequence of the oligosaccharides determines the antigen specificity. For example, a self with alleles A and O has the blood antigen A with transferred GalNAc. Galactose (Gal), fucose, and GalNAc, the three carbohydrates that are linked to protein and lipid in the red blood cell membrane, serve as the minimum immunological determinants for the blood antigen A against which mainly IgM subclass of anti-A isoantibody is raised in non-selfs who have blood type B antigen carrying Gal instead of GalNAc. IgG type of anti-A and anti-B isoantibodies are more frequently detected in individuals who have blood type O bearing neither GalNAc nor Gal. Those non-selfs were never immunized by type A blood cells from a self.

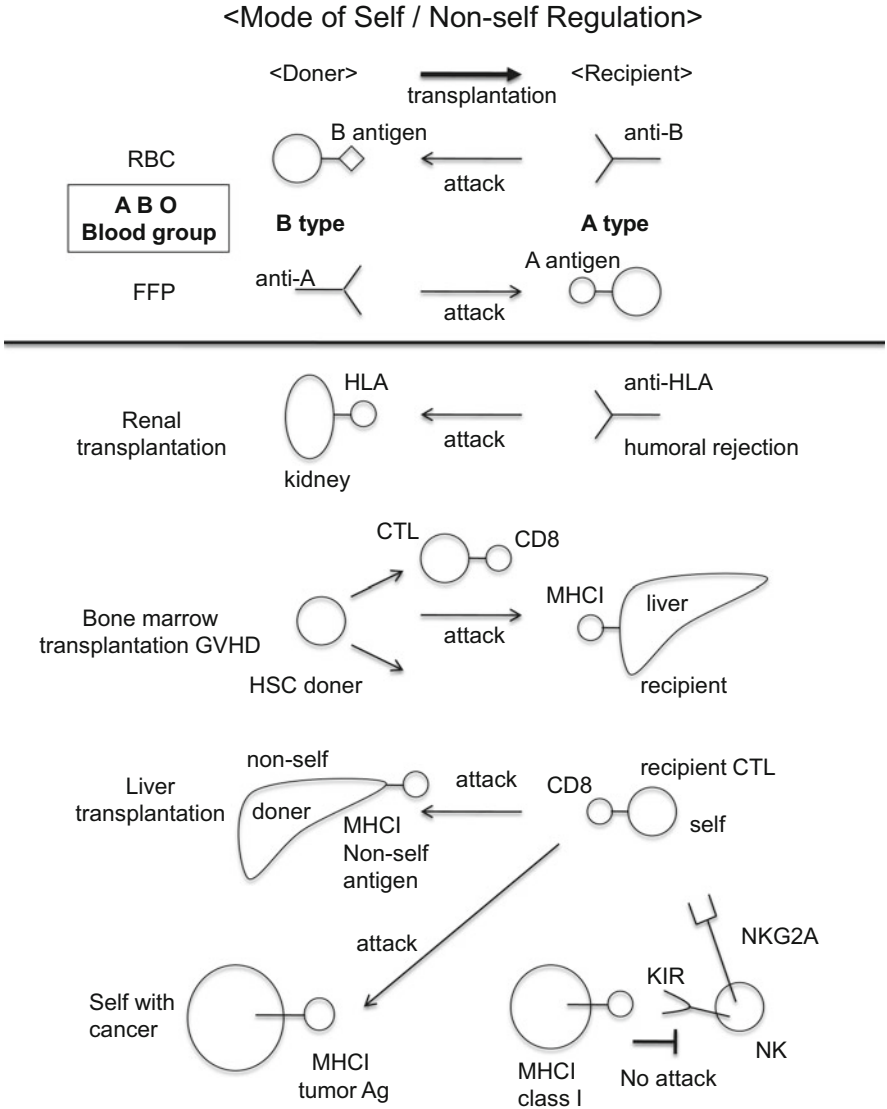


Fig. 5.5 Iso-antigen recognition. NK cells attack MHC class I-non-bearing cells. Even if MHC I is present, the cells are attacked by CD8⁺ CTL in conditions in which MHC I is non-self or is coexpressed with non-self tumor antigens. Fresh frozen plasma (FFP) transfusion often is applied in clinical settings. If FFP from type B donor is applied to mismatched recipients cells with A antigen, the cells are attacked. KIR : killer cell Ig-like receptor

Given that the antibodies are usually absent at birth but are present in most subjects by 6 months, the sugar moieties taken from food or bacteria are implicated in the immunization [47]. The similar but pathological circumstance is described as a

molecular mimicry theory in the pathogenesis of Guillain-Barré syndrome (see Chap. 4). This suggests that some environmental forces switch the non-self from microbes, for example, to human beings. Although the mechanisms are not yet defined, the AB type beings are thought to be more resistant to cholera than the O type. This divergence may confer some opportunities to survive against bacterial assaults on human. The other functions of those glycosyltransferases remain to be elucidated. However, it is of note that a genomic statistical analysis revealed a close association between the ABO blood types and serum levels of E-selectin essential for inflammatory leukocyte recruitment in the atherosclerotic lesions.

Fetus with blood type A is protected against anti-A isoantibody of mother with blood type B, because the IgM type cannot pass through placenta. IgM is efficiently triggers complement cascade for hemagglutination responsible for hemolytic transfusion reaction. Because IgG subtype of isoantibodies that possess the ability to transverse placenta often are found in blood type O subjects, hemolytic disease of the newborn may take place when group O mother has a group A, B, or AB child.

The blood antigens are expressed not only in red blood cells but also in epithelial cells and endothelial cells. Whether blood transfusion or kidney transplantation, mismatched ABO blood type gives rejection reactions through binding of isoantigen and isoantibody with complements. Because donors are more limited in the latter than the former, immunosuppressive therapies, including calcineurin inhibitors, are applied in the latter. In mismatched transfusion of unfortunate occurrence, acute hemolytic reaction takes place within 24 h.

5.6 Major Histocompatibility Complex

The supergene major histocompatibility complex (MHC) encompasses a 7-Mb region on human chromosome 6p21 with dense clustering of more than 300 genes [48]. The gene products from the well-studied human leukocyte antigen (HLA) and non-HLA genes, such as the inflammatory cytokine, $\text{TNF}\alpha$, and MHC class I chain-related gene A (MICA), which is a ligand for NKG2D receptors in innate immune response, work in concert to govern self-recognition (see Fig. 1.3 in Chap. 1). Currently, even with optimal donor-recipient HLA matching, afflictions potentially due to non-self recognition based on non-HLA variation on the recipient side can happen, including graft-versus-host disease (GVHD) and transplant rejection.

The discovery of HLA began in 1952 with a small experiment by Jean Dausset in which he mixed the serum from an agranulocytic woman with the bone marrow of another individual and found a macroscopic agglutination, leading him to think of the presence of allo-antibodies [49]. The classical HLA molecules responsible for antigen presentation consist of 26 class I and 24 class II gene products, and reversely, the $\text{TNF}\alpha$ gene is located in the class III region. The fourth players involved in antigen presentation include CD4 and T-cell receptor (TCR) on naïve T cells and MHC class II molecule and non-self antigen epitope coupled to it, for

example derived from virus, on antigen-presenting cells, such as DC and macrophages. Another fourth players of self-recognition include CD8 and TCR on cytotoxic T lymphocytes (CTL) and MHC class I molecule and self-epitope peptides derived from protein turnover of the cell on all nucleated cells, such as endothelial cells of engrafted kidney.

In renal transplantation, monitoring of isoantibody in recipient against donor HLA has been extensively performed with success. If you have a positive reaction in donor lymphocytes mixed with recipient serum by adding complement, activation of complement cascade eventually makes a pore in the cell membrane to death, which can be easily recognized in the presence of dye that stains the dead cells. This is called lymphocyte cytotoxicity test (LCT) or complement-dependent cytotoxicity (CDC) test and routinely utilized in clinical settings. These days detection of complement non-fixing isoantibodies by flow cytometry is being performed, because not all donor-specific antibody (DSA) can fix complement (flow cytometry cross-match, FCXM) [50]. Even if FCXM is positive, desensitization by infusion of a large amount of immunoglobulin could change it from positive to negative providing recipient with a chance for renal transplantation.

A study with 315 renal transplants with no pretransplant DSA showed that 15% patients acquired de novo DSA in mean years of 4.6 with lower 10-year graft survival of 57% compared with 96% in those without de novo DSA. The mechanism of generation of DSA in pretransplant recipient is not perfectly clear as in the case of ABO blood type.

In GVHD e.g., bone marrow transplantation, transplanted hematopoietic stem cells repopulate and differentiate into immune cells, which target recipient organs in a manner opposite to rejection reaction in renal transplantation. A variety of cytokines are produced during GVHD. Antibody-based targeting CD132, which is the γ chain common to receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 reduced production of TNF α and IL-6 and improved patient survival [51].

In murine transplantation models of allograft, transfusion of donor splenic leukocytes (DST, donor-specific transfusion) give a synergy with blocking antibody against CD154 (also called CD40L) to induce prolonged survival or tolerance in allograft [52]. CD154 is a costimulatory ligand for CD40. A negative costimulation is provided by the CTLA4-B7 binding. Allograft survivals were not changed under DST with leukocytes derived from CD40-KO, MHC-I, and II-double KO or B7-KO mice. However, recipients were deficient in MHCII and the prolongation was suppressed, suggesting the indirect antigen recognition by CD4⁺ T cells. TLR activation by either CpG or LPS could abolish the costimulation blockade. Conversely, both TLR4-KO and MyD88-KO mice prolonged allograft survival. Likewise, CpG promoted graft rejection by inhibiting Treg and facilitating Th1 differentiation.

5.7 Tumor Immunology and Vaccine

Is tumor a non-self? If a given non-self provides immunogenic antigens on a self, the antibodies against them may eliminate the non-self. Tumor cells arise within an individual not transplanted from other ones. In this regard, nothing could be non-self to the tumor-bearing self. However, suppose that tumor could change the blood type O gene in a self by its genetic instability to blood type A, then the endogenously generated anti-A isoantibody that is already present in the self would be able to attack tumor cells as perfectly as red blood cells in mismatched transfusion. However, given the critical single base deletion in the O gene from the A gene, it may be beyond redemption to recover both coding frame and activity of the enzyme by additional deletion or insertion. The story is not so simple in tumor cells. Tumor cells have at least 11 hallmarks to survive (see Part III).

Tumor-specific antigen to show non-self properties for the self as host has been poorly defined in most tumors, and immunotherapy has not achieved reasonable success. The most promising situation is found in nonmyeloablative (NMA) allogeneic stem cell transplantation in chronic myeloid leukemia (CML) [53]. All CML cells express the BCR-ABL oncoprotein that is not found in physiological conditions. Therefore, it can be interpreted that BCR-ABL is recognized as foreign or non-self in cells that express it, or those BCR-ABL-expressing cells are non-self for other normal cells in the host as a self. By NMA transplantation, donor leukocytes immunologically attack all leukemic cells in the recipient. This is called graft versus leukemia (GVL) effects and is a leukemic version of graft versus host disease (GVHD) in which donor organs or cells attack the recipient. The opposite attack is called rejection reaction. In both cases, the attack is executed between self and non-self. Even in this successful instance, it is unclear how normal donor cells can eliminate recipient malignant cells simply because BCR-ABL oncoproteins are localized in the cytoplasm not on the surface of leukemic cells to be immunologically recognized. Still, I believe that it is possible that non-self features of tumor cells, if any and if present, can exert immunological elimination programs by the self. How tumor cells escape immune-surveillance is discussed in the tumor section of Part III.

Genetically different markers can allow the donor-derived cells that were included in the organ or tissue of the donor to be traced after transplantation to recipient. In addition to the well-reported, donor-derived leukemia in the recipient after BMT, epithelial neoplasms can arise after allogeneic kidney transplantation. Analysis of 21 skin squamous cell carcinomas (SCC) in kidney transplantation recipients uncovered R175H and Q61K mutation in p53 and K-Ras, respectively, in the SCC, and only the p53 R175H also was detected in the recipient kidney 7 years before the SCC diagnosis [54]. This indicates that cells that underwent allogeneic transplantation are not only targeted by the immune system but also, conversely, can give rise to tumors by acquiring genetic mutations.

5.8 Concluding Remarks

A variety of substances of exogenous origin, such as those from microbes and plant, have their own endogenous receptor(s) in host exposed to them. The receptor recognizes non-self and activates immune system. Transplantation of an organ from a different individual and development of tumors within his own body can be recognized as presence of non-self that is originated from self. Therefore, irrespective of its origin, non-self can be sensed by endogenous receptors.

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Chapter 6

Extension of Endogenous Field

6.1 Overview

Conversion between self and non-self can affect the field size for each. There are several ways by which the conversion is achieved.

The T-box transcription factor Brachyury is essential in epithelial to mesenchymal transition (EMT) of cells during mesodermal development, which the word EMT of the primary tumor cells as the first step of metastasis originates from [1]. Brachyury is aberrantly expressed in tumor cells but not normal adult tissues. The restricted expression of Brachyury in terms of time and cell types can provide an opportunity to target this early embryonic antigen by the CD8⁺ T cell-mediated immune system [2]. In this case, the self-protein Brachyury may no longer be self when expressed in tumor cells in adults and can be targeted as a non-self protein. I suppose that there are at least three more modes by which self can be transformed to non-self. First, changes in quantity of self. Second, structural changes of self. Third, untolerated changes in the localization of self.

Let us think of uric acid for another example involved in a human disease. This is a final product of DNA metabolism and actively secreted in urine by organic transporters in kidney. Its physiological function is still unknown. When serum concentration of uric acid increases, it forms a crystal structure (monosodium urate <MSU> crystals) in articular cavity due to its pH-dependent chemical property [3, 4]. This results in the well-known disease, gout, which is an inflammation of the joint mediated by the NOD-like receptor (see Table 2.1 in Chaps. 2 and 8 for details) in synovial cells [5]. Vascular permeability and neutrophil infiltration in the inflamed joint cause swelling and pain. The elevated amount, soluble form to crystal, serum to intra-articular mislocalization, aforementioned all changes take place.

Venomous snakes, such as cobra and crotalus (rattlesnakes), are not absolutely protected against or immune to the action of their own venoms. This means that the self-protein, e.g., α -neurotoxin by itself, is harmful to the same self by binding to its

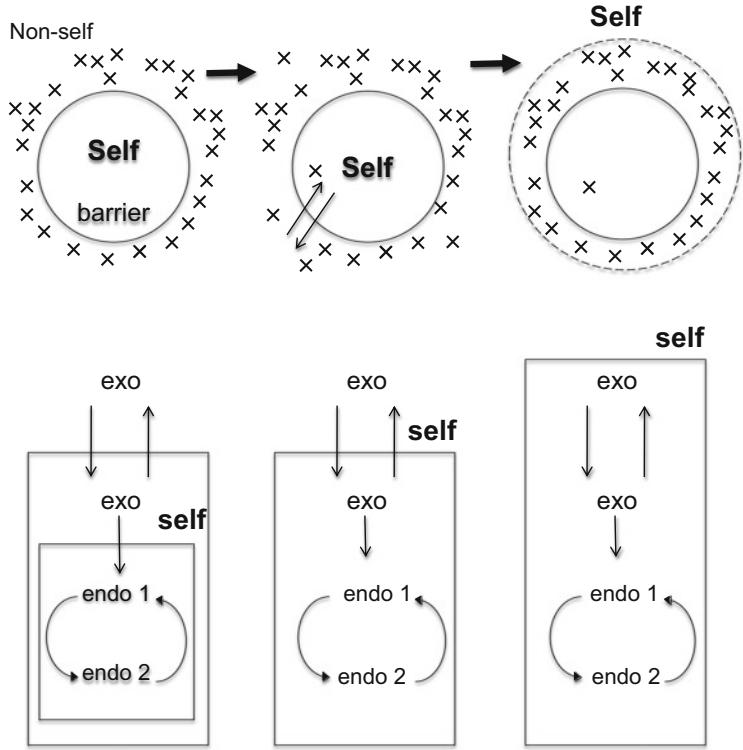


Fig. 6.1 Conceptual extension of self. We carry *E. coli* (non-self, many x) in our colon that fail to induce colitis by physical as well as immunological barriers in the intestinal mucosa. If you consider the low concentration of LPS (non-self, corresponding to single x within the barrier) in the portal vein as one of the self factors that function in a homeostatic manner in our body, the whole *E. coli* may be considered to be an extended self

own nicotinic acetylcholine receptor (nAChR). This example for the idea of “correct position” indicates that there must be a watertight barrier or boundary between α -neurotoxin produced and stored in a modified version of salivary gland and nAChR in the neuromuscular system within the same self. If the barrier is not tight enough and allows a small amount of leakage of the toxin that is not sufficient to be lethal, some defending system, such as endogenous inhibitors for the neurotoxin or immune responses, could be established, and the snake may fail to be a victim of its own toxin. Both skin and intestinal barriers against microbes present a clear picture of invasion of non-self (microbes) into self (host). However, as far as homeostasis is concerned, self often cannot do without non-self to establish a homeostatic milieu (Fig. 6.1).

When one thinks of the opposite, non-self could be assumed to belong to self. For example, we carry approximately 10^{12} bacteria, such as *Staphylococci*, in the skin and 10^{14} , including a variety of species that generate LPS in the gastrointestinal tract (GIT). Although the luminal side of the GIT starting from mouth to anus

is ostensibly inside of the body, it is actually outside of it and houses bacteria that are taken for granted as foreign. The homeostasis of GIT cannot be maintained without those non-self. Then, does the true self include bacteria?

6.2 Changes in Quantity

Overexpression of S100A8 (self) could be misrecognized as LPS (non-self), both of which are sensed by TLR4. This is discussed in detail in Part IV.

Here, I introduce molecules that are present in physiological conditions but elevated in pathological circumstances to cause host responses. The well-known example is advanced glycation end product (AGE). Glucose is a reducing sugar that exists in solution under equilibrium between a cyclic form closed by a single oxygen atom and an open chain form to reveal a functional aldehyde group. The glycation reaction, alternatively called Maillard reaction, is a nonenzymatic reaction between the aldehyde group of reducing sugars and amino residues of proteins. In the case of glucose and hemoglobin consisting of hetero-tetramer of α and β globins with heme, the amino-terminus of β globins forms a Schiff base, an intermediate with a carbon-nitrogen double bond, subsequently converted to an amadori product, which is named HbA1C and routinely used to monitor the serum glucose levels in diabetic patients. Depending on the protein targets of glycation reaction, AGE varies, for example from tissue to tissue. Because glycation by itself deteriorates the functions of the target proteins, AGE binds to scavenger receptors, such as CD36, to be physically eliminated from the body as too much non-self products. Beyond clearance some but not all species of AGEs, including carboxymethyl lysine (CML), transmit intracellular signaling through receptor for AGE (RAGE). RAGE is expressed everywhere but at low levels except for its abundance in lungs and up-regulation in inflammatory lesions. Soluble forms of RAGE that are cleaved by MMP9 and ADAM10 but still retain the binding ability to AGE are found. They can serve as a decoy receptor and participate in functional elimination of AGE.

RAGE signals via MAPK and NF κ B with resulting up-regulation of VEGF, VCAM-1, TNF α , IL-1 β , IL-6, CCL2, and so forth. I suppose that readers can be reminded of a similarity to the LPS – TLR4 signaling. As described in the following chapters, S100A9 and HMGB1 are claimed to bind RAGE. Given that those molecules accumulate in diabetes as well as inflammatory disorders and could signal through TLR4, it may be understood that application of soluble forms of RAGE protected mice against diabetic neuropathy-related pain more efficiently than RAGE knockout.

6.3 Changes in Quality

6.3.1 *Crystal*

Structural transformation can generate molecules that are not usually present in physiological conditions. The most representative example is crystals and amyloid fibrils, both of which apparently acquire longer half-life in the host body than each of their soluble counterparts in the serum or tissues. Not only aforementioned MSU but also amyloid- β has been shown to activate NLRP3 inflammasome.

6.3.2 *Amyloid β Fibril*

First of all, I will briefly define the term <amyloid> to avoid confusion. Amyloid is generic name to indicate insoluble fibrillar proteins [6]. The amyloid β of Alzheimer's disease is a peptide of 36–43 amino acids proteolytically processed from the amyloid precursor protein (APP), which is integrated in the membrane of synapses of neuron with an as yet unknown function. Accordingly, a variety of peptides that are irrelevant to APP are known to be able to self-assemble into amyloid fibrils containing arrays of β -sheets running parallel to the long axis. The highly ordered structure of amyloid β fibril is opposite to the intrinsically disordered structure of proteins, such as BCR of the BCR-ABL oncoprotein found in human chronic myeloid leukemia [7]. Experimentally, the structural conversion or fibrillar structure of amyloid can be monitored by transmission electron microscopy, circular dichroism (CD) spectroscopy, and conveniently by the binding to a benzothiazole dye thioflavin T, which exhibits enhanced fluorescence on binding along the fibril. The secondary structure of proteins includes α -helices, β -strand, β -turn, and disordered regions, which exhibit unique wave length-dependent dichroism. The overall structural transition, e.g., during aggregation of amyloid β fibrils, can be monitored by CD values. An in vitro system to develop aggregates has been established by which to observe time-dependent fibril formation. Interestingly, intermediate states of aggregates, prefibrillar amyloid, but not its mature form, of peptides even irrelevant to Alzheimer's disease also could be toxic to neurons by disturbing cellular Ca^{2+} and redox conditions, suggesting inherent toxicity of aggregates. Clinically, accumulated fibrillar peptides of amyloid β are found in senile plaques in Alzheimer's disease. The consistent finding of higher expression of IL-1 β in the surrounding microglia and cerebrospinal fluid as well as the presence of bone marrow-derived leukocytes led to the discovery that individually knockout mice of NLRP3 or Caspase-1 were rescued from manifestations of Alzheimer's disease, such as memory loss. The amyloid β -activated NLRP3 induces expression of neurotoxic TNF α and NO in an IL-1R-dependent manner suggesting auto- or paracrine mechanisms of cytokine expression [8, 9]. The

NLRP3 dependency also was found in the expression of chemokines, including CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CXCL2 (MIP-2).

Accordingly, structurally transformed amyloid β is no longer self, and this non-self protein originated from the self is recognized cytoplasmic pattern recognition receptor NLRP3 to exert innate immune responses. Amyloid A fibrils derived from serum amyloid A1 and A2 (SAA1 and SAA2) bound to a soluble form of RAGE with a Kd 60.3 nM.

S100A9 is up-regulated in brain of both Alzheimer's disease patients and Alzheimer's disease model mice Tg2576. S100A9 facilitates fibrillization of β amyloid, which induces S100A9 expression in microglia [10]. The impaired learning and memory were improved 2 months after injection of lentiviruses expressing anti-S100A9 shRNA into the hippocampus [11].

S100A8 also can form amyloid body. The details of S100 family of proteins, including S100A8 and S100A9, will be the focus of Chap. 15.

6.3.3 Oxidized Products

Oxidation sometimes changes the property of molecules. I discuss oxidized phospholipids (OxPLs) that are found in apoptotic cell membranes, lipid-bearing macrophages in subendothelial space in fatty streak lesions of atherosclerosis, and pulmonary surfactants. Oxidation of 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (OxPAPC) gives a mixture of dozens of different OxPLs among which 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) are actually detected in atherosclerotic lesions. When assessed in vitro with cultured human pulmonary artery endothelial cells by transendothelial electrical resistance (TER) and endothelial integrity as judged by images of VE-cadherin, OxPAPC at low dosage of 10 μ g/ml increased endothelial barrier function but decreased at high dosage of approximately 100 μ g/ml [12]. OxPAPC at 50 μ g/ml is equivalent to 62.5 μ M provided that its mean MW is 800.

Binding between LPS and any one of the three binding proteins, LBP, CD14, or MD-2, was inhibited by OxPAPC but not PAPC (Fig. 5.2 in Chap. 5) [13–15]. The antagonistic function of OxPAPC was observed in the LPS-stimulated NF κ -B activation and expression of IL-8 and E-selectin mRNAs at 10–60 μ M when tested in HUVEC [16]. OxPAPC alone can induce expression of IL-8, CXCL1, and CXCL3 but not LPS response gene products, including IL-6, TNF α , and E-selectin. Interestingly, the antagonistic activity for the LPS-induced IL-8 expression, but not E-selectin, changed to agonistic at high dosages over 100 μ M. E-selectin also is induced by TNF α and IL-1 β , which is not affected by OxPAPC. Thus, given that OxPAPC targets MD-2 for the E-selectin expression, OxPAPC is a full antagonist against LPS leaving no room for a partial agonistic activity (see below for the pharmacological view). Both totally synthetic POVPC and PGPC are capable of inhibiting TLR4-mediated NF κ B signaling and E-selectin mRNA expression.

LPS induces production of ROS, which in turn oxidizes PLs. The conversion of PLs as self to OxPLs as non-self is supposed to provide an endogenous negative feedback with signaling by LPS as non-self.

The chemical composition of lung surfactant is roughly phospholipid in 90 %, neutral lipid such as cholesterol in 5 %, and surfactant proteins (SP-A, B, C and D) in 5 %. The major phospholipid is saturated di-palmitoyl-phosphatidylcholine and unsaturated phospholipids, including PAPC. The functions of pulmonary surfactant include lowering the breathing effort on inspiration and anti-inflammatory activities. Upon intratracheal instillation of HCl or inactivated H5N1 influenza viruses, lung histological analyses revealed the appearance of OxPAPC in a manner dependent on p47, an activating component of the superoxide producer Nox2 complex. Synthesized and oxidized OxPAPC but not untreated PAPC induced acute lung injury with elevated production of IL-6 when administered intratracheally dependently on TLR4 and TRIF. The other elevated cytokines included IL-1 β and IL-8 but not TNF α , G-CSF, etc. The same group of researchers also showed the dependency in macrophage stimulation experiments in vitro. The OxPAPC at 10 μ g/ml stimulated induction of IL-6 at >250 pg/ml in lung tissue macrophages in a manner dependent on TLR4 and sensitive to eritoran, a TLR4/MD-2 complex inhibitor.

This is in disagreement with what I have just described. The former group showed that OxPAPC stimulated HUVEC at 30 μ g/ml to produce no IL-6. We also had no positive results in our hands. I assume that the levels of IL-6 induction by OxPAPC are basically low and may not be a direct effect. The above-mentioned observation is complicated, and I will try to explain it on the basis of pharmacology.

Pharmacological View of Partial Agonist

It is valuable to discuss the pharmacological points of view of agonist and antagonist not only to deepen the understanding but also to obtain insights about the apparently complicated phenomenon of OxPAPC. Someone claims that its activity depends on TLR4 and others pose its inhibitory activity on TLR4.

In pharmacology, the assumption is that a drug binds a receptor. It is supposed that receptor status is in equilibrium between inactive and active conformation. A drug that has a higher affinity to active conformation will shift the balance to active state, thus activating the receptor. This drug is called an agonist. The complete preference to the active conformation gives a full agonist. If an agonist binds less to the inactive conformation than the active one, it is called a partial agonist. If a drug that fully binds the inactive form and its pharmacological effect is opposite to what is exerted by an agonist, it is called an inverse agonist. This is the situation in which to explain the relationship between one agonist and one receptor.

When one drug blocks the effect of the other drug, it is called an antagonist. If a drug binds receptor without causing any effect but blocks the

(continued)

activity of an agonist, in other words in the presence of the agonist, the drug is called a full antagonist. The following situations are slightly more complicated. If a drug by itself works as an agonist when applied alone and the same drug has a blocking activity against another agonist when they are present concomitantly, namely working as an antagonist, it also is called a partial agonist (Fig. 6.2). Examples of partial agonists include buprenorphine,

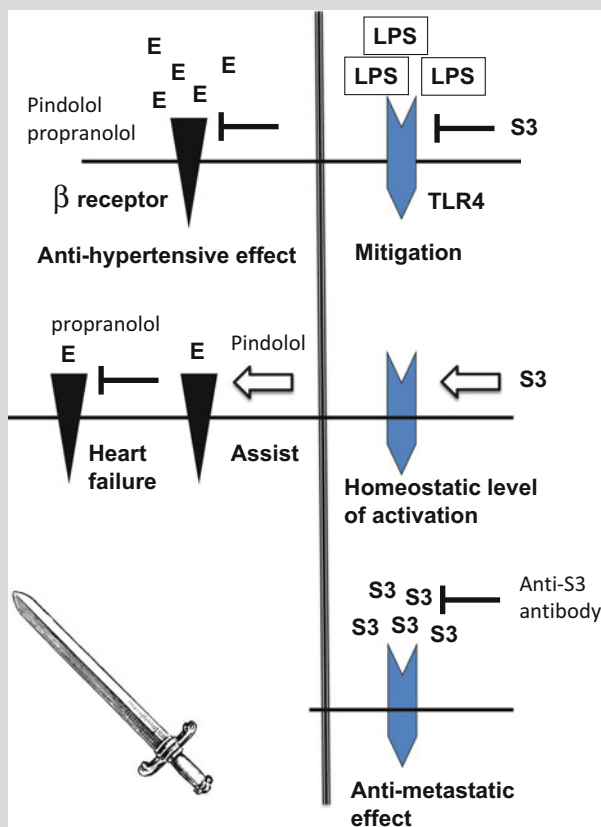


Fig. 6.2 Partial agonists. Examples of epinephrine (E) and SAA3 (S3) (see Chap. 15). Patients with hypertension have β adrenergic receptor activation by excess E, which can be suppressed by β antagonists such as pindolol and propranolol. During sleep, for example, blood pressure may fall down owing to reduced E. If the patient takes propranolol, hypotension and potential heart failure could take place. In this situation, another β -blocker pindolol functions as a partial agonist and works as an agonist. This effect is called intrinsic sympathomimetic activity. Too much SAA3 was shown to promote establishment of pre-metastatic microenvironment in lungs serving as an endogenous TLR4 ligand (agonist) (see Chap. 15). Conversely, in excess of LPS, it blocks the TLR4 signaling (antagonist). In the absence of LPS signaling, in other words in sterile conditions, SAA3 could activate TLR4 in a homeostatic level (agonist)

pindolol, and aripiprazole. It is worth explaining each of them, because the idea is useful in Part 4.

Exogenous morphine of botanical origin causes analgesia and addiction through MOP(μ) receptor. Buprenorphine alone also induces analgesia via MOP(μ) in naïve individuals. However, it shows an antagonistic function in the presence of morphine. Therefore, the analgesic effect is reduced when it is used in combination with morphine, and the antagonistic effect causes withdrawal symptoms when applied to the addicted subjects. Because the respiratory depression effect is low with buprenorphine, it is useful for detoxification of morphine. Needless to say, a full antagonist naloxone also is useful for the same purpose, because it has only antagonistic but no agonistic function. When targets of blockade have endogenous but not exogenous (such as morphine) origin, we need to consider the activity of endogenous agonists.

At least two dopamine signal systems are known in the midbrain. The first is the nigrostriatal dopamine system, which has cell bodies of dopamine neurons in substantia nigra that project to neostriatum, in which tonic stimulation with low concentrations of dopamine in the synaptic cleft is sufficient to inhibit the onset of Parkinson's disease, which is caused by degeneration of this hypersensitive system. The second dopamine neurons are localized in the ventral tegmental area projecting to ventral-striatum, which forms the mesolimbic system. This hyposensitive system requires high concentrations of dopamine for phasic activation after rewards and reward-predicting stimuli [17]. Hyperactivation of the second system is believed to cause positive symptoms in schizophrenia. D2 inhibitors, such as chlorpromazine, block both systems causing drug-induced Parkinsonism. The classical antipsychotic drugs bind dopamine receptor D2 with antagonism. Aripiprazole, an atypical antipsychotic drug also does so with high binding ability to D2. However, it binds the G protein-coupled state (active form) of dopamine receptor D2 with twofold higher affinity than uncoupled (inactive form) D2, consistent with its nature as a partial agonist. While it shows antagonistic activity in the presence of high levels of D2 activation, it also serves as a partial agonist when the D2 activation levels are low, thus stabilizing the D2 activation status. The classical D2 inhibitors show extrapyramidal side effects, such as Parkinsonism, by too much D2 inhibition, but aripiprazole has a low propensity for the side effect presumably due to the balancing effect. The well-known β -blocker pindolol and acebutolol inhibit β adrenergic receptors in the presence of much epinephrine, for example, in the daytime when life activity is high thereby serving as antihypertensives. During sleep when activity is low with less epinephrine, they serve as an agonist for the β receptor. This is considered to be useful to avoid too much inhibition of adrenergic activity aggravating

(continued)

heart failure. This agonistic effect is called intrinsic sympathomimetic activity (ISA).

There is one more idea called a mixed agonist–antagonist. Pentazocine works as an agonist for morphine receptor MOP(k), causing analgesic affects when used alone or for naïve subjects. When applied to patients addicted to morphine, it functions as an antagonist for MOP(m) to block morphine effect, resulting in withdrawal symptoms. The point is that the partial agonist can bind a different receptor that binds the other agonist against which it antagonizes.

With those pharmacological views in mind, let us think about the effects of OxPAPC. We should bear in mind that we discuss the pharmacological way of understanding the relationship between an agonist plus or minus an antagonist and given readouts as a pharmacological effect without solid evidence of which receptor physically binds OxPAPC. When the pharmacological outcome or effect is the E-selectin mRNA levels, OxPAPC is a full antagonist against LPS as far as MD-2 binds OxPAPC. LPS also can induce expression of IL-8. When the effect on mRNA levels of IL-8, CXCL1, and CXCL3 is concerned, OxPAPC is a partial agonist for LPS especially at low concentrations. However, at high concentrations, the antagonistic activity changes to agonistic, suggesting the activation of different receptors from TLR4.

6.3.4 End Products of Lipid Oxidation

The most representative and bioactive species is 4-hydroxynonenal (HNE) [18]. It can react with DNAs and cysteine residues of proteins. The serum concentration in normal subjects ranges 0.3–0.7 μM but increases to 20 or even higher in local microenvironment under oxidative stress. A good thing is that this transformed non-self is short-lived, and it is reported to have the half-life of only 5 min in hepatocytes. It can activate receptor tyrosine kinases, such as EGFR, and transcription factors, including Nrf2, involved in expression of antioxidant genes by targeting cysteine residues of its cytoplasmic suppressor Keap1.

6.3.5 Too Much Degraded Extracellular Matrix

In response to tissue injury that causes inflammation, activities to restore the tissue to the normal state are necessary and actually exist in self-limited circumstances. One of the triggering mechanisms is pathological degradation of extracellular matrix components, such as collagen, fibronectin, and hyaluronan. Their chemical

structures and enzymes responsible for the degradation are described in details in Part III. Here I pick up fibronectin and hyaluronan to explain the idea that self is converted to non-self to give an alarm to the tissue for repair.

6.3.5.1 Fibronectin

Fibronectin is a large, disulfide-bonded, hetero-dimer glycoprotein with each subunit of 210–230 kDa. In inflamed synovial fluid in rheumatoid arthritis patients, a variety of fragments ranging from 30 to 200 kDa are found to be elevated. Many sorts of proteases are capable of the proteolytic cleavage at least in vitro, including thermolysin, plasmin, and thrombin. One of the unique features of fibronectin is that degraded products generated this way either lose or acquire stimulatory activities compared with full-length or rest of the fragment, such as fibrin-binding ability (see Chap. 3). Another intriguing property of fibronectin is its alternatively spliced variant containing the region called EIIIA (the extra domain A encoding type III repeat). The EIIIA-containing fibronectin is induced during tissue injury in a TGF β -stimulated fashion [19]. The 160 kDa variant fibronectin purified from human placenta lacked the 12th type III repeat and carboxyl-terminal sequences at least partially exposing the EIIIA domain and was capable of stimulating rabbit synovial cells to produce MMP-1. The recombinant EIIIA fragment proteins produced and purified from *Escherichia coli*, which was claimed to contain LPS less than 0.6 pg/ μ g protein, could induce expression of TNF α , IL-1 β , and IL-6 in a TLR4-dependent manner.

Naming of Sugar-Related Molecules

Glycan is a poly- or oligo-saccharide. Proteoglycan is a covalent combination of protein and sugar (glycan refers to sugar, namely oligo- to poly-saccharide residues), especially GAG. GAG, previously called mucopolysaccharide, is a polymer of disaccharide containing hexosamines, such as galactosamine, including hyaluronan, chondroitin sulfate, keratan sulfate, and dermatan sulfate. When protein part constitutes the main structure, it is called glycoprotein.

6.3.5.2 Hyaluronan

Hyaluronan is one of the major glycosaminoglycans (GAG) family of carbohydrates to constitute extracellular matrix in tissues where remodeling is active [20]. GAG is a systemic name (like sucrose vs. cane sugar) of what was previously called mucopolysaccharide and consists of linear and repeated disaccharides containing hexosamine. GAG includes chondroitin sulfate, heparan sulfate, dermatan sulfate, heparin, and so forth. Hyaluronan is a nonsulfated polymer

greater than 1,000 kDa with repeated disaccharides of D-glucuronic acid and N-acetylglucosamine under physiological conditions. It is synthesized by at least three enzymes, hyaluronan synthase (HAS) 1, 2, and 3 [21]. Although only HAS3 can synthesize small hyaluronan of approximately 200 kD, larger hyaluronan can be degraded to the similar sizes. Artificially prepared hyaluronan of 135 kD by HCl starting from *Streptococcus hyaluronan* of 1,500 kD, which was claimed to have LPS levels <40 pg/mg of the hyaluronan, could stimulate expression of CXCL2 in a manner dependent on MyD88 and combination of TLR4 and TLR2, and independent of the authentic receptor CD44 [22]. The activity was sensitive to hyaluronidase.

In the resolution phase of the tissue injury-induced inflammation, CD44 is required for elimination of the degradation products of hyaluronan. Because in the bleomycin model of lung injury with CD44 knockout mice, low molecular weight hyaluronan accumulation was observed. Hyaluronan is endocytosed by binding to CD44. Their persistent presence causes unremitting inflammation in this model.

This implicates that <non-self>, which is the degraded and thus pathologically transformed hyaluronan, if not cleared, continues to stimulate the surrounding microenvironment, which is <self> capable of recognizing the existence of non-self. Low molecular weight hyaluronan of 200 kDa, which was purified by fractionation from the serum of adult respiratory distress syndrome patients, stimulated murine peritoneal macrophages for CXCL2 (MIP-2) mRNAs expression in a manner dependent on TLR2, TLR4, and MyD88.

In both bleomycin-induced and hyperoxia-induced lung injury, murine models with O₂ > 98%, mice doubly knockout for TLR2 and TLR4 were more susceptible than the wild-type mice. Although what is essentially responsible for repair is not clear, because the monitored products derived from TLR-MyD88 signaling are chemokines that stimulate emigration of leukocytes with double-edged swords, they claimed a protective role by non-self products hyaluronan based on those experiments.

Mucopolysaccharidosis (MPS) is caused by defect in enzymes involved in GAG catabolism, resulting in GAG storage that induces inflammation. For example, MPS VII mice have facial deformity, short femur length, and increased serum levels of TNF α , which were normalized when bred to TLR4-KO mice.

6.4 Beyond Barrier

6.4.1 DNAs in the Cytoplasm and Sensor

The location of DNAs in eukaryotic cells is strictly limited to their nucleus and mitochondria. If aberrant DNAs are encountered in the cytoplasm or extracellular space, DNases efficiently digest and clear them in physiological settings. However,

when such DNases as DNase I are mutated with impaired activity, accumulation of incompletely digested DNAs causes TLR-independent production of IFN β and inflammatory responses [23]. Although nucleic acid sensor proteins for microbial RNA and DNA are studied extensively as described in the previous chapter, it remains to be elucidated what mechanisms exist to recognize endogenous DNAs of aberrant location.

Extracellular DNAs are sensed by TLR9 in endosomes, whose threshold was reported to be lowered by RAGE promoting the DNA uptake [24]. RAGE-KO mice failed to recruit neutrophils in the lungs when a low dosage of CpG DNA was intranasally administered.

6.4.2 *Intra- to Extracellular Transit*

6.4.2.1 ATP

The simplest molecule that gives alarm or regulatory signals to the surrounding microenvironment and eventually to the whole body is ATP [25]. In healthy conditions, intracellular concentration of ATP generated in mitochondria is in the millimolar range. When released into extracellular space, whether physiologically or pathologically, its half-life is very short—measured in seconds due to surrounding hydrolytic activities. Therefore, even in pathological settings, such as necrotic cell death that passively releases ATP, its target cells need to be in a limited paracrine radius. However, only nanomolar concentrations of ATP are sufficient to stimulate purinergic signaling. In physiological conditions, ATP is actively secreted from hepatocytes when their cell volume increases and restores the volume to the hepatocytes through activation of Cl⁻ channels. This regulation in cell volume homeostasis is achieved by exocytosis- and channel-dependent ATP secretion to the outside of cells. Accumulation of fatty acid in the liver inhibits the exocytosis resulting in the deterioration of cell volume.

Extracellular ATP serves as DAMP (see Chap. 4) by binding its receptor P2X7 to induce a variety of intracellular signaling, including activation of NLRP3 inflammasome in which K⁺ efflux is required (see Chap. 8).

6.4.2.2 Heme

Heme, which is highly toxic due to generation of ROS by spontaneous auto-oxidation and intercalation to the cell membrane due to its nature, is released during hemolysis, rhabdomyolysis, or clot formation, which is subjected to clearance by heme-scavenging systems, such as (1) haptoglobin (Hp) that binds hemoglobin (Hb) and promotes phagocytosis of the Hb-Hp complex by macrophages via CD163, and more importantly (2) hemopexin that captures heme with high specificity. Hemopexin (Hx) is an acute phase reactant produced in liver. Hemopexin

directs heme to hepatocytes and macrophages where the heme-Hx complex is internalized via LDL receptor-related protein-1 (LRP-1, also called CD91) to undergo hemoxygenase (HO)-1-mediated catalysis yielding CO and biliverdin and Fe [26]. It is of note that most human MMPs contain the hemopexin-like domain in the carboxyl-terminus responsible for various functions (see Chap. 11).

When those scavenging capabilities are overwhelmed by the heme accumulation, oxidative burst could take place. Experimentally, while peritoneal macrophages stimulated by heme at 30 μ M showed ROS production in a TLR4-independent manner and TNF α secretion in a manner dependent on TLR4, MyD88, or CD14, but sensitive to serum that should contain hemopexin, intraperitoneal injection of heme at 60 μ g per cavity induced neutrophil infiltration in the cavity in a TLR4-independent manner but failed to induce plasma TNF α elevations. Heme could hardly show adjuvant activity and TLR4-dependent secretion of TNF α by heme was insensitive to E5564 (eritoran), a lipid A analog. Collectively, heme could activate TLR4 in a different mechanism from LPS.

6.4.2.3 HMGB1 and HMGN1: Alarmin Concept and Danger

The high mobility group (HMG) family of proteins was initially discovered as constitutively expressed chromatin-associated nonhistone proteins in the eukaryotic nucleus [27]. The amino acid sequence is highly conserved with 99% identity between rodents and human. The family consists of HMGB (alternatively called HMG1 or amphoterin), which binds the linker regions of the distorted DNAs, HMGA (HMG-I/Y), which binds the minor grooves of A/T-rich DNAs, and HMGN (HMG-14/17), which binds nucleosomes. They bind DNAs in a structure-dependent manner. HMGB1 has two homologous motifs called A- and B-box containing three α -helices with the latter domain responsible for binding distorted DNAs. HMGB1 competes with histone H1 and implicated in the regulation of transcription factors, including glucocorticoid receptor. HMGB1 knockout mice die immediately after birth due to wasting presumably by lack of the steroid hormone effects.

Although HMGB1 was found to induce neurite outgrowth, it is likely that HMGB1 is not absolutely required for at least the early stages of neurogenic development. HMGB1-knockout cells are hypersensitive to psoralen plus UVA irradiation due to the poor ability to eliminate UV-induced CPDs and 6-4PPs (see Chap. 10 for details) from the damaged DNAs. HMGB1 has been implicated not only in nucleotide excision repair as just mentioned but also in mismatch repair, base excision repair, and double strand break repair [28]. However, despite its binding property to DNA and repair factors, accurate roles in DNA repair system remain uncovered.

In addition to the roles in transcription and DNA repair, the third function was discovered in 1999 [29]. When in vitro-cultured macrophages were stimulated by LPS, HMGB1 was actively secreted more than 8 h after stimulation. It is of note that early LPS mediators, such as TNF α and IL-1 β , are usually released within 5 min. Also in cells undergoing necrosis, HMGB1 is passively released into the

extracellular microenvironment. The recombinant full-length HMGB1 and its B-box fragment protein purified from *E. coli* bind the TLR4/MD-2 complex with Kd of 1.5 μ M and 22 μ M, respectively. Synthetic peptides of 20-mer within the B-box containing the Cys106 stimulated macrophages to produce TNF α . However, a mutated peptide in which the Cys 106 is changed to Alanine lost the activity (see Chap. 7 for details).

In summary, HMGB1 is pooled in the nucleus to serve in DNA homeostasis before both active and passive release upon LPS assault or during necrosis. Even in the absence of LPS, necrotic cell-stimulated sterile inflammation involves HMGB1. Purified HMGB1 when injected to C3H/HeJ mice (500 μ g per mouse) that is LPS-resistant due to a mutation in TLR4 was lethal within 16 h, suggesting that HMGB1 elicits lethal signaling through different receptor(s) from TLR4, including TLR2 and RAGE.

HMGN1 directly binds and activates poly (ADP-ribose) polymerase-1 (PARP-1) that is engaged in recognition of damaged DNA lesions and in the DNA repair [30]. HMGN1 knockout conferred resistance against a DNA-alkylating reagent methyl methanesulfonate (MMS) on sensitive cells. Apart from its role in DNA transaction, HMGN1 also functions extracellularly through TLR4 in dendritic cell maturation [31]. Given that EG7 mouse thymoma cells overexpressing ovalbumin (OVA) as a tumor-associated antigen produced fewer OVA-specific CD8 T cells in HMGN1-KO, both HMGN1 and HMGB1 also could serve as an immune-adjuvant (see below).

6.4.2.4 Intra- to Extraluminal Transit

In murine animal models using leptin-deficient C57BL/6J ob/ob (obesity model mice, in which the leptin gene involved in energy homeostasis, is disrupted) mean serum LPS levels in the portal vein are documented to be 95 and 184 pg/ml, respectively, prominently higher than those being 51 pg/ml in normal control mice in an experimental condition [32]. By showing a negative correlation between the LPS levels and electrical resistance of intestinal specimens as well as mucosal permeability of horseradish peroxidase, they claimed that low levels of inflammation that is accompanied by elevated serum concentration of TNF α in those mice weakens the resistance of intestinal mucosal barrier by disrupting the integrity of tight junctions allowing the entry of LPS into circulation. In other experiment with apolipoprotein (apo) E knockout mice (ApoE mediates binding of apoE-containing lipoprotein, such as VLDL to LDL receptor essential for circulating cholesterol clearance, and therefore its knockout mice serve as a good animal model of atherosclerosis), serum LPS levels were approximately 100 pg/ml, which is increased to more than 300 pg/ml by a high-fat diet in the background of second gene knockout of IRAK.

In humans, circulating LPS in serum is detected in physiological conditions in the range of 150–350 pg/ml [33]. Chromium ^{51} Cr, labeled *E. coli* endotoxin when injected intravenously into rabbits showed a rather short serum half-life of 90 min

with preferential accumulation in liver, lung, and spleen (Fig. 8.2 in Chap. 8). Intravenous administration of LPS in volunteers at approximately 3 ng/kg showed elevations of TNF α and IL-8 peaked at approximately 2 h, IL-6 at 4 h, and CRP within 24 h. Of note, IL-1 β was not detected. Assuming that BW is 50 kg, the extrapolated serum concentration of the injected LPS at least at the time of injection is approximately 80 pg/ml, which is supposed to be superimposed on the baseline concentration of LPS already present in the serum before injection. Serum LPS has been reported to increase significantly in normal, healthy subjects eating high-fat, high carbohydrate meals. In severe periodontitis patients, in which epidemiological studies have revealed close linkage with carotid artery stenosis and periodontal bacteria, such as *Porphyromonas gingivalis*, can actually be detected in the carotid artery plaques, the serum LPS levels increase and correlate with those of serum TNF α at LPS concentration >870 pg/ml, suggesting another origin of LPS not only from intestine but also gingiva).

Thus, in both humans and mice, certain low levels of LPS, which is non-self if present in the intraluminal side of GIT, are in fact within self without causing any particular diseases. Then, what is the biological significance of those low dosages of LPS in host? As a phenomenon, a high-fat diet that actually passes through the GIT appears to facilitate the entry of LPS into the serum by disrupting the GIT barrier.

Here I distinguish between <low> (50–100 pg/ml) vs <high> (more than 10 ng/ml) LPS for discussion [34]. The word LPS without any adjectives to indicate its dosage means <high> in this book.

In bone marrow-derived monocytes (BMDM), whereas high or usual dosages of LPS for experimental use induced transient expression of IL-6 mRNAs, which peaked at 5 h after stimulation and back to baseline levels in 10 h, low dosages of LPS induced persistent elevation of IL-6 mRNAs, which did not form a peak and its levels were retained even after 10 h after stimulation. Of note, the highest expression levels of IL-6 mRNAs in high versus low LPS were hundreds of times higher in high dosages. The IL-6 induction, as well as that of CCL2, was almost totally abrogated in BMDM that were derived from IRAK knockout mice [35]. The researchers who reported those results also showed that cytokine induction is dependent on Tollip, ROS, and cell surface TLR4 and is accompanied by the activation of ATF2 and C/EBP δ . This was consistent with the results of the animal studies mentioned above where increased serum levels of IL-6 and CCL2 in ApoE knockout mice on high-fat diet were restored to baseline levels in ApoE-IRAK double knockout.

Intraperitoneal (ip) injection of LPS induced increased blood monocyte frequency as monitored by CD11b⁺Ly6Chigh, which concomitantly took place with a decrease of the same population of cells in the bone marrow and peaked 4 h after injection. The peak of the LPS dosage was 20 ng per mouse, but a low dosage of 100 pg per mouse significantly induced the increased number of blood monocytes as high as 15 % in the ratio of Ly6Chigh relative to CD45⁺ cells from the baseline level of 5 %. Neutrophil frequency failed to make a peak of LPS dosage. This phenomenon was dependent on MyD88, TLR4, and CCR2 as judged by knockout mice for each. Bone marrow cells that responded to LPS to produce CCL2 were identified as CD45⁺, Ter119⁺ (erythroid marker), Sca1⁺, CD31⁺, PDGFR β ⁺, and

CXCL12⁺ cells that were localized in perivascular regions. Experiments that compared ip versus intravenous (iv) administration of LPS revealed no difference to achieve the induction levels of plasma IL-6 approximately 1000 pg/ml. Given that ip-injected LPS needs to enter the blood to stimulate those bone marrow cells directly, the above-described observation is based on low dosages of LPS.

Therefore, it is likely that low dosages of non-self LPS is integrated in self and serves as a continuous stimulator for low levels but persistent expression of at least IL-6 and CCL2 (Fig. 6.1). This contributes to the formation of GIT barrier between non-self and self, not allowing an entry of increased levels of LPS. Utilization of non-self (LPS) not in the recognition of non-self (LPS) but in the homeostasis of GIT barrier implicates that the actual self consists of original self plus small amounts of non-self in the interface.

6.5 Concept of Adjuvant: Linkage to Acquired Immunity

Non-self is not invariably immunogenic. Morphine or colchicine of botanical origin binds its receptor MOP (μ) or β -tubulin, respectively, in humans to exert valuable clinical effects. However, repeated usage in clinical settings seldom raises antibody against them.

The immunogenicity has been believed to be one of the criteria for non-self. However, efficient production of antibody can be achieved by co-delivery of helper molecules called adjuvant (adjuvare in Latin means to aid or help). This immune-potentiator is usually lipid alone or in combination with molecules of microbial origin as represented by the well-known Freund's complete adjuvant containing oil emulsion and inactivated *Mycobacterium tuberculosis*. It can aid in the prolonged survival of the antigen and stimulation of antigen-presenting cells, such as dendritic cells and macrophages. When utilized in vaccine, it can reduce the amount of the injected antigen and therefore is useful for economical and practical reasons.

The most commonly used adjuvants include aluminum hydroxide and paraffin oil, but monophosphoryl lipid A (MPL) and its homolog are used in vaccines for human papilloma virus and influenza virus [36]. MPL is thought to be a TRIF pathway-specific agonist of TLR4. Those compounds are clearly non-self and supposed to be recognized by the pathogen sensors that activate innate immunity.

6.6 Endogenous Adjuvants

Subcutaneous injection of ovalbumin to mice elicited an increase of ovalbumin-specific IgG, which is drastically enhanced when co-injected with purified recombinant HMGB1 with <0.03 EU per mouse (see Chap. 7 for LAL test details) [37]. Host TLR4 was dispensable as judged by similar levels of immunization between C3H/HeN and C3H/HeJ mice. The latter mice have a loss-of-function

mutation in TLR4. This indicates that the adjuvant activity in this experiment depends on neither TLR4 nor contaminating LPS. HMGB1 is a nucleosome-binding protein that remains bound to chromatin in apoptotic cells but is released from necrotic cells. Apoptotic RMA lymphoma cells are a poor immunogen and therefore tumor developed when transplanted with living lymphoma cells 14 days after the apoptotic cell injection. But when apoptotic cells were co-injected with supernatants of necrotic fibroblasts from wild-type but not HMGB1 knockout mice, anti-lymphoma immunological rejection took place. This indicates that nuclear HMGB1 is self but released HMGB1 from necrotic cells is a transformed self to non-self, serving as an adjuvant.

The most widely utilized adjuvant in clinical settings is aluminum hydroxide (Alum) [38]. Intraperitoneal injection of ovalbumin with Alum as an adjuvant potentiated DC activation whose three fundamental steps were molecularly analyzed: antigen processing, presentation to naïve T cells, and migration to draining lymph nodes (DLN). There is an experimental system in which ovalbumin conjugated with BODIPY fluorochrome (OVA-DQ) gives green signals when taken up by cells and processed in acidified lysosomes and red signals during enrichment in endosomal antigen processing compartment. Intraperitoneal injection of OVA-DQ induced double positive mDC ($F4/80^+MHCII^+CD11c^+$) in the peritoneal lavage more abundantly when co-injected with Alum. In CD11c⁺ cells, expression levels of MHCII, CD86, and CD40 were positively correlated with DQ signal levels, indicating that Alum potentiated DC maturation. Sorted Alum-treated mDC was more growth-promoting for naïve OVA-specific T cells than untreated. The taken-up OVA is processed into immunogenic fragments in the DLN, which could be measured by DQ-positive cells and were 17-fold enhanced by Alum. Mediastinal LN drains both lungs and peritoneal cavity. The CD11c promoter-linked and diphtheria toxin-dependent system to deplete conditionally CD11c highly positive cells was executed by administration of the toxin. Intraperitoneal but intratracheal administration abolished the T-cell growth in mediastinal LN, indicating that the responsible LN drains the mDC from the peritoneal cavity when both antigen OVA and adjuvant Alum were injected, because while the intratracheal method eliminates only resident DC the intraperitoneal affects DC systemically.

Then, what is the mechanism? Alum by itself failed to mature bone marrow-derived DC to mature DC. The basic mechanisms involve induction of uric acid from necrotic cells in the peritoneal lavage, which promoted strong neutrophil influx with expression of CXCL1 (KC), CCL2, IL-1 β , and IL-18. Although the authors did not show the expression of HMGB1 in those experiments, any compounds or procedures that cause cell death especially necrosis should be able to serve as an efficient immune-potentiator by inducing endogenous ones. UA activates NLRP3 inflammasome (see Chap. 8).

6.7 Concluding Remarks

Conversion between self and non-self can be caused by changes in time, intracellular localization, intra- versus extracellular localization and luminal versus abluminal localization. Integration of non-self into the self field results in extension of the field.

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Chapter 7

Evidence for Existence of Endogenous TLR4 Ligands

Before focusing on the discussion of the endogenous ligands of TLR4—accurately speaking the TLR4/MD-2 complex—we are absolutely in need of the knowledge of its authentic ligand, LPS. In human, circulating LPS in serum is detected in physiological conditions in the range of 150–350 pg/ml.

7.1 Definition

Generally, ligand simply indicates a molecule(s) that binds specifically to a receptor. For example, acetylcholine (Ach) is a ligand. It has three subtypes of its own receptor (AchR-M1 to M3) of muscarine action. Both Ach and M2, for example, are generated endogenously. Pharmacologically, bethanechol, which is synthesized in vitro and therefore not produced in host cells, also binds M2 to exert a similar effect to Ach. Then, bethanechol is called an agonist, discussed in the previous section (pharmacological view), capable of working in place of Ach of endogenous origin. In this context, Ach and bethanechol are an endogenous and exogenous ligand, respectively, of M2. Butylscoporamine instead blocks the Ach action and is called an antagonist. Because butylscoporamine competes with Ach for binding M2, it also is an exogenous ligand for M2.

7.2 Exogenous Versus Endogenous Ligand, Which Is Discovered First?

Needless to say, hepatocyte growth factor receptor HGFR (Met) binds its authentic endogenous ligand HGF. Interestingly, a protein called internalin B (InlB) of Gram-positive microbe *Listeria monocytogenes* was found to bind HGFR (Fig. 5.1 in

Chap. 5) [1, 2]. Although InlB binds at two different interfaces from that of HGF as judged by co-crystal structure analysis, it can activate HGFR by inducing receptor clustering and subsequent endocytosis. The levels of MAPK activation and kinetics of the disappearance of HGFR from the cell surface are similar to each other. This is an example in which structurally different ligands derived individually from self and non-self can directly bind their receptor in the self at separate sites but elicit the same signaling. Given that deregulated HGFR signaling facilitates cell growth and metastasis, the promiscuity in the ligand in *Listeria monocytogenes*-infected cancer patients may result in progression of tumor.

In the case of TLR4 of endogenous origin, its authentic ligand LPS of exogenous origin has never been called an exogenous ligand, because it is needless to express its bacterial origin and endogenous counterpart has never been in the mind of researchers in this field. This is because TLR4, one of the essential sensors in innate immunity, is believed solely to recognize potentially harmful exogenous or foreign molecules for the host to defend against invading organisms. This historical circumstance has made a slight confusion of those terms. Without knowing the presence of ligand of endogenous origin, LPS was utilized just like an endogenous ligand, such as Ach. LPS is not a valuable drug for clinical usage but is certainly a pharmacological reagent that can serve as an agonist if authentic endogenous ligands exit in host cells. If an endogenous ligand can block the LPS effect, it is now called endogenous antagonist. If TLR4 binds a new molecule of exogenous origin in addition to LPS, it is called an exogenous ligand, agonist, or antagonist. Both LPS and paclitaxel of exogenous origin have been shown to bind MD-2 to activate the TLR4/MD-2 complex (see Chap. 5).

7.3 Candidates of TLR4 Ligands

As shown in Table 7.1, many molecules are claimed to be potential TLR4 ligands. Some of them are assumed due to the requirement of TLR4 for the biological activity to be exhibited by the ligands (necessary condition), and others are tested in their ability to bind TLR4 directly (sufficient condition) or indirectly depending on the experimental methods. I do not intend to be a second-round reviewer of the already evaluated and published elaborate works. However, with the given information in those excellent papers, I will point out how to understand their significance by comparing each and the leave choice of whether to believe to the readers.

Here are two points by which to make a logical judgment:

1. TLR4 and/or MD-2 are necessary for the ligand activity.
2. The ligand is sufficient to bind TLR4 or MD-2.

Table 7.1 Claimed host-derived TLR4 ligand candidates. This list never guarantees that any of them, including Tenascin-C [41], is a true endogenous TLR4 ligand. As stated, scientific levels of the evidence provided by each claimer should be assessed by readers. I provide one way to do it in this book. From an evolutionary point of view, some of them have interspecies variations. For example, granulysin is a highly cationic protein found in human CTL and NK cells but no homologous protein in mice. It not only activates cell migration and production of TNF α and IL-6 in both human and murine DC in a manner dependent on TLR4 but also serves as an adjuvant to mediate anti-ovalbumin antibody generation [43]. SAA3 proteins are found in mice but not in humans according to the medical literature, but we found unique isoforms (see Chap. 15)

TLR4	Host derived ligands	Ref.
1	S100A8	[20]
2	SAA3	[21]
3	HMGB1	[12]
4	Decorin	[8]
5	Biglycan	[22]
6	CD138	[23]
7	α -crystallin A chain	[24]
8	β -defensin 2	[25]
9	Endoplasmin (Gp96)	[26]
10	Fetuin-A	[27]
11	Fibrinogen	[28]
12	Fibronectin	[29]
13	Heparan sulfate	[30]
14	HSP22	[24]
15	HSP60	[31]
16	HSP70	[32]
17	HSP72	[33]
18	Hyaluronan	[34]
19	Monosodium urate crystals	[35]
20	OxPAPC	[36]
21	PAUF	[37]
22	Prx1	[38]
23	Resistin	[39]
24	SP-A	[40]
25	Tenascin-C	[41]
26	Saturated Fatty Acid	[42]
27	Granulysin	[43]
28	heme	[44]

7.4 Question of Method

How to consider the coexistence of LPS, other lipids, or proteins with the experimentally prepared candidate ligands.

7.4.1 *Limulus Amebocyte Lysate (LAL) Test*

To prove that your candidate of TLR4 ligand, whether agonist or antagonist, actually can stimulate cells that express the TLR4 in the presence or absence of MD-2, it is required that your ligand preparation is devoid of LPS contamination. Irrespective of the source of preparation, whether experimental *Escherichia coli*, yeast, baculovirus, or mammalian cells, LPS contamination is unavoidable. Even

chemically synthesized peptides or other molecules, which are theoretically deficient in LPS, need to be dissolved in certain solvents before stimulation of in vitro cultured cells. As you can imagine, heavy contamination of LPS in solvent water, for example, could give similar results to what are obtained with LPS. Therefore, LPS contamination needs to be examined logically in any experimental preparations.

Because LPS contamination is detrimental in clinical settings, the U.S. Food and Drug Administration has provided a standard by utilizing the *Limulus* ameobocyte lysate (LAL) test (see Chap. 5). The quantification of LPS by the enzymatic reaction in the coagulation system of this ancient arthropod (see details in the previous Chap. 5) is expressed as ten endotoxin units (EU) equivalent to 1 ng of LPS. A test kit from Wako, Japan, uses a different *E. coli* standard and 1 ng LPS equals to 7 EU. Since fungal β -glucan also gives a positive signal in the *Limulus* reaction, the reaction mixture is saturated with a large amount of β -glucan for the readout to be specific to LPS. The detection limit is 0.21 mEU/ml by the Wako kit. According to the Guidance for Industry for endotoxin testing provided by FDA in 2012, the LPS limit for approval is 0.25 EU/ml (25 pg/ml) for drugs in general. Although the prototypical LPS from *E. coli* is hexa-acylated, tetra- or tri-acyl lipid A exhibits high activity in LAL test but inactive in the cytokine production in mammalian cells.

7.4.2 *Is Polymyxin B Reliably Effective to Remove LPS?*

Polymyxins were discovered in 1947 [3]. Polymyxin B (PMB) is a lipopeptide metabolite produced by Gram-positive rod *Bacillus polymyxa* found in plant roots in soil nutritionally helping the plant. It is a mixture of more than 30 components with two major: polymyxin B1 and B2. It was not until the recent clinical use as a last-line antibiotic that its application has been hampered by adverse effects, such as nephrotoxicity. However, polymyxins are under revival of investigation to develop drugs efficient for emerging infections caused by multiresistant, Gram-negative bacteria, especially *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and so forth.

The mechanisms of bactericidal effects by polymyxins involve direct binding to lipid A in LPS and subsequent permeabilization of outer membrane of Gram-negative bacteria. LPS located in the outer membrane has an amphipathic (= amphiphilic) structure consisting of both hydrophilic and hydrophobic (= lipophilic) properties [4, 5]. Based on the three-dimensional NMR solution state structure of the PMB-LPA complex, an electrostatic interaction between negatively charged phosphate group in lipid A and positively charged Dab residues (diaminobutyric acid, which is non-proteinogenic [not found in the genetic code] but is a component of bacterial cell wall) in polymyxin B. This is followed by the hydrophobic interaction between the short fatty acyl tail of polymyxin B and fatty acid leaflet of LPS.

In vitro studies of polymyxin B and LPS by isothermal titration calorimetry, which can provide rather accurate kinetic information of binding of molecules by measuring calorie generated on their interaction, as well as concomitant X-ray

diffraction analysis showed an approximately 1:1 binding stoichiometry with a dissociation constant K_d around 1.5 μM and aggregate structures of LPS changes from cubic to multilamellar in the presence of polymyxin B with the latter being inactive in stimulation of $\text{TNF}\alpha$ production in human mononuclear cells but still positive in LAL test. In chromatography-based removal of LPS, including affinity columns with polymyxin B and anion exchange columns, is currently utilized and effective.

7.4.3 Boiling

It is an alleged notion that heat can inactivate proteins by denature but LPS is heat-resistant. Based on the idea, a peptide candidate for endogenous TLR4 agonist should lose its activity by boiling for example. If the activity is retained as in the case of LPS as a negative control, the preparation is supposed to be contaminated with LPS or some heat-resistant active molecules. However, there is a report that a low dosage of LPS at 50 pg/ml even after heating at 100 °C for 1 h was still capable of inducing IL-6 in mouse bone marrow-derived dendritic cells. If IL-6 mRNA levels are used as a readout for TLR4-mediated stimulation experiments by the candidate protein and its preparation contains other proteins capable of inducing IL-6 induction, proofs to show absence of LPS in the preparation are not sufficient. Boiling should be able to eliminate the activity, which is misinterpreted as inactivation of your protein of interest. You need to show the absence of stimulators that are usually, but not always, proteins. A sensitive ELISA or Western blot with a good antibody is a reasonable experiment.

7.4.4 Believe or Not

How do we interpret the unavoidable contamination of endotoxin in the preparation of candidates by purification from bacterial expression system? If you read an article whose materials and methods provide information saying that LPS levels of certain proteins purified from *E. coli* are <0.1 EU/ml (10 pg/ml) by LAL test, you would think that it is even below what is recommended by the FDA, and therefore, you believe in the quality of the preparation. Suffice it to say that everybody in the world should be able to reproduce the experiment. Based on the activity of the candidate protein, biochemical and biological results should be logically provided. A report exists that shows the following details. LPS (*E. coli*; Serotype R515. TLR grade from Enzo Life Science) at low dosages of 10 or 20 pg/ml can still induce IL-6 (ELISA from BD Pharmingen) or $\text{TNF}\alpha$ (R&D system) secretion, respectively, in mouse bone marrow dendritic cells. Statistical significance of $p < 0.001$ required minimum of 50 pg/ml LPS for both of the cytokines. In this system, preincubation of 100 $\mu\text{g/ml}$ of polymyxin B (from Sigma) with 1 pg to

1 $\mu\text{g/ml}$ LPS revealed that quenching effects of polymyxin B is insufficient at LPS dosages higher than 50 pg/ml [6].

7.5 Good Exemplars for Demonstration

Next, I will discuss some of the good evidence to prove that a candidate molecule is an endogenous ligand for the TLR4/MD-2 complex. There is so much accumulating evidence in literature to prove the point, but I suppose that convincing demonstration should be supported by consistent results of experiments from the standpoint of (1) biochemical, (2) cellular, and (3) animal model studies. I believe that even if you find a molecule that binds TLR4 in vitro lack of corresponding significance in biology not only depreciates the value of discovery but also puts the in vitro evidence under skepticism.

Structurally speaking, LPS is essentially a combination of sugar and lipid, lipopolysaccharide (see Fig. 2.5 in Chap. 2). Both biglycan and decorin consist of sugar and protein. SAA3, S100A8, S100A9, and HMGB1 are proteins. OxPAPC is a mixture of lipid. Gb4 is glycolipid in which oligosaccharide is attached to a lipid ceramide. Therefore, good candidates could be sugar, lipid, protein, or their combination.

7.5.1 *Biglycan and Decorin*

Biglycan and decorin belong to a family of small leucine-rich proteoglycan (SLRP) that comprises 17 genes [7]. Both contain core protein of approximately 40 kDa with 55 % identical amino acids that are covalently attached to a side chain(s) of glycosaminoglycan (GAG) (see Note in Chap. 6) of either chondroitin sulfate or dermatan sulfate at the amino-terminal serine residue(s); biglycan has two, decorin has just one. Dependent on the connective tissues where biglycan and decorin are expressed, they have a different sugar moiety. For example, biglycan in articular cartilage has two dermatan sulfate side chains and decorin in bone has one chondroitin chain. They participate in the stabilization of ECM, such as collagen. In cartilage destruction in osteoarthritis, biglycan, but not decorin, is a good substrate for MMP-13 and is cleaved in the middle of the core protein. Heterozygotes for a frame shift mutation of decorin have been documented to suffer from corneal opacities. Secreted biglycan can be detected culture media of rat mesangial cells, which is down-regulated by stimulation by IL-1 β .

I will describe the evidence published in literature and information kindly provided by Liliana Schaefer (Pharmazentrum Frankfurt, Institut für Allgemeine Pharmakologie und Toxikologie) (Figs. 7.1 and 7.2).

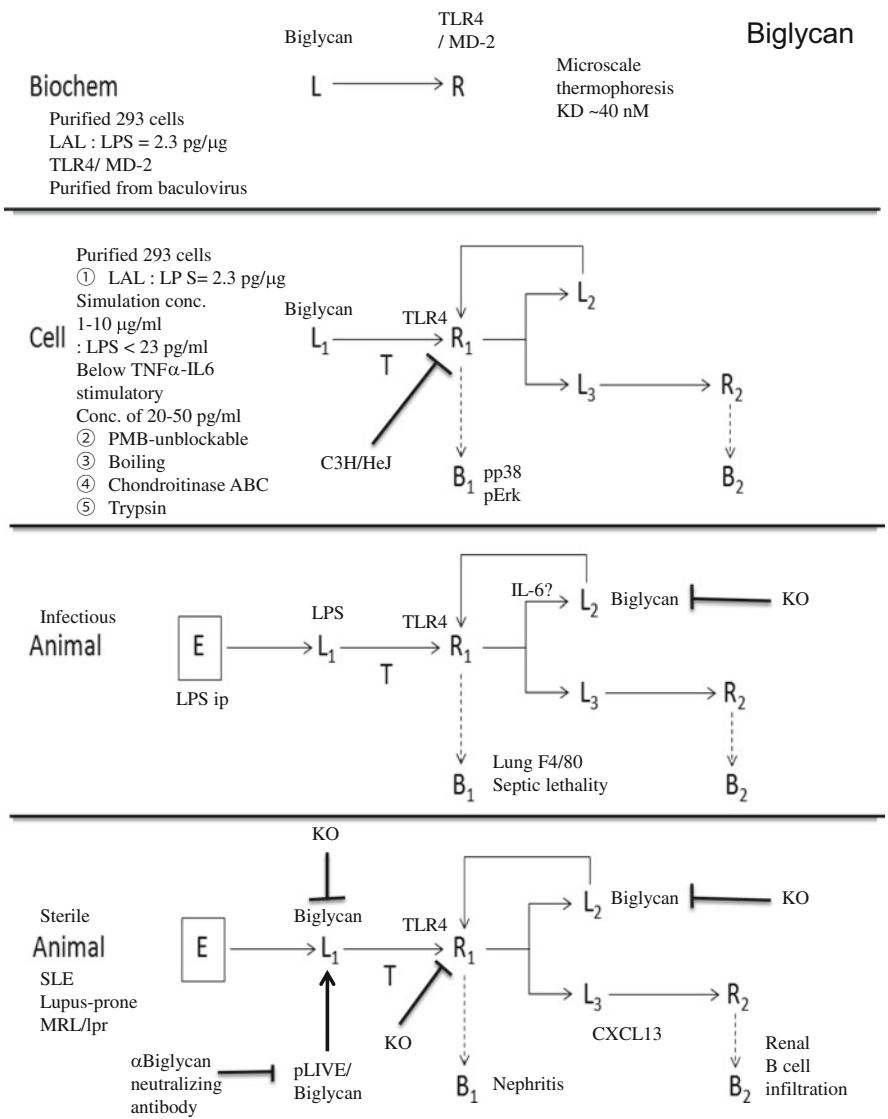


Fig. 7.1 Evidence for biglycan. Ligand (L) is biglycan and receptor (R) is the TLR4/MD-2 complex. Evidence is summarized from the standpoint of biochemical (Biochem), cellular (Cell), and in vivo (Animal) levels. For example, by showing contaminating LPS amounts by LAL test, biglycan purified from mammalian 293 cells induced TNFα expression with activation of p38 in vitro, which could be inhibited by PMB, boiling, or TLR4 mutation background (C3H/HeJ mice). In an animal model of infection, intraperitoneal (ip) injection of exogenous (boxed E) induced expression of the endogenous TLR4 ligand (L1) biglycan (L1) presumably mediated by IL-6 and L1 feedback onto TLR4 for amplification of the signaling resulting in lethality by sepsis. In a sterile inflammation model of SLE, nephritis was inhibited by knockout of TLR4 or biglycan

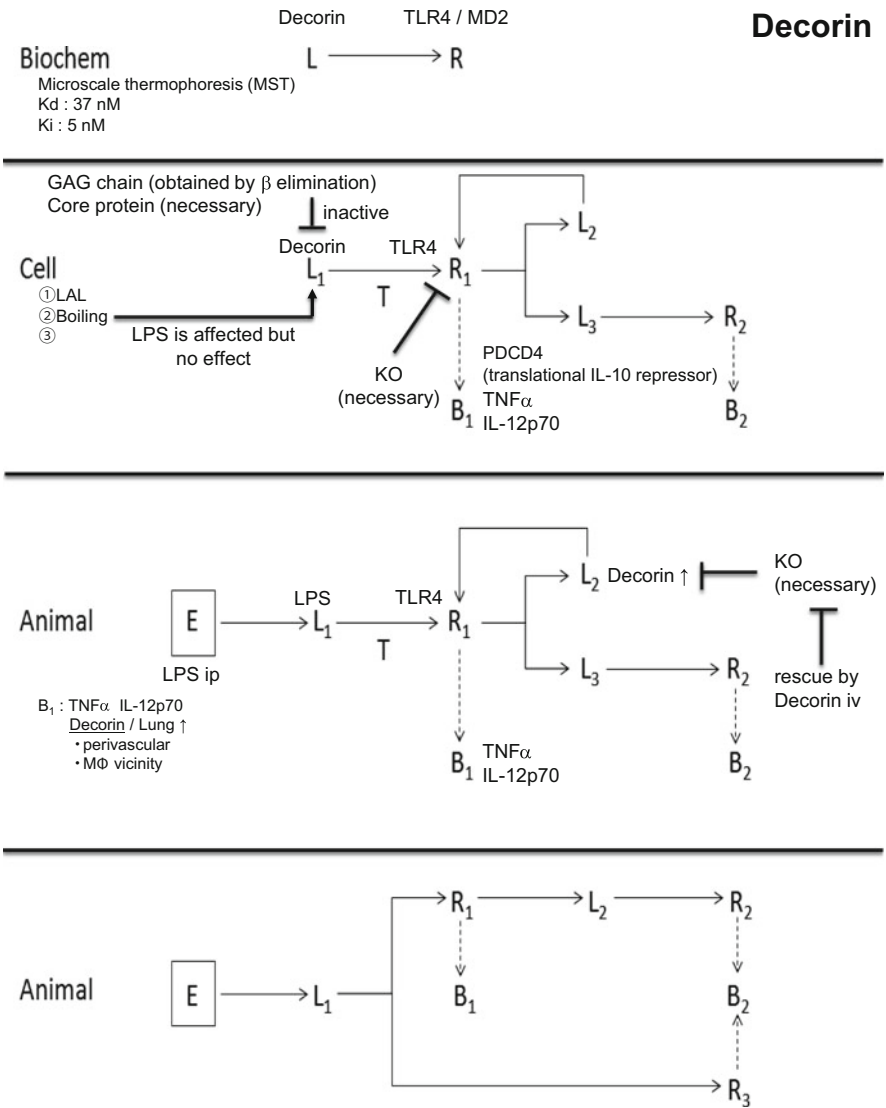


Fig. 7.2 Evidence for decorin (See Fig. 7.1 legend for biglycan)

7.5.1.1 Biochemical Aspect

Due to the sugar chains, biglycan and decorin have prominent poly-anionic characteristics. Another structural feature is the central presence of ten leucine-rich repeats (LRRs) flanked by cysteine-rich regions, which is common to all SLRP members. It should be remembered that all TLR family members have LRR in the extracellular domain. The potential functions of LRR in general are described in

details in Chap. 5. Briefly, LRR in a protein can bind other LRR of a different protein.

Decorin purified from mammalian cells binds the TLR4/MD-2 complex with K_d 37 nM as judged by microscale thermophoresis and biglycan behaves the same way with a similar K_d [8]. Decorin directly binds TLR4 but not MD-2, suggesting that the binding is mediated by LRR. In fact, the core protein is sufficient to bind TLR4. However, GAG or core protein alone is not sufficient, and both are obligatory for TLR4 to execute intracellular signaling.

7.5.1.2 Cellular Aspect

Based on the results of LAL test with purified biglycan giving 23 EU/mg of biglycan, the calculated concentrations of contaminating LPS in stimulation experiments by biglycan were 3–30 pg/ml, which is under the concentration high enough to induce TNF α expression. In combination with polymyxin B that is reported to be effective with LPS < 50 ng/ml (see the aforementioned information on the effectiveness by polymyxin B), the research group showed no difference in the capability to activate p38 and pErk in the presence or absence of polymyxin B. The activity was lost after boiling, which should inactivate at least the protein core by denature inhibiting the binding to TLR4. Collectively, all three fundamental procedures—LAL test, polymyxin B, and boiling—are reasonably used to prove the point.

In addition, chemical modifications of biglycan by chondroitinase ABC treatment that digests GAG or trypsin digestion to eliminate the protein core could inactivate the stimulation activity. By utilizing cells from C3H/HeJ mice that have defective TLR4 by genetic mutation, it was shown that TLR4 is necessary for the activity. As in the case of LPS, biglycan can activate NLRP3 inflammasome (see Chap. 8) by clustering TLR4 and P2X receptors [9].

7.5.1.3 Animal Studies

Intraperitoneal injection of LPS, which mimics infection of Gram-negative bacteria, induced septic lethality with increased number of F4/80-positive macrophages in the lungs. LPS-stimulated TLR4 promoted production of endogenous mediators, including IL-6, which induced biglycan. Biglycan activated TLR4 by positive feedback providing amplification of the storm of endogenous mediators. This phenomenon is rescued by biglycan gene knockout.

In a noninfectious condition, e.g., lupus-prone mice, biglycan is up-regulated to cause nephritis with renal infiltration of B cells. By doing the experiments in the background of either TLR4 knockout or biglycan knockout, both of them were demonstrated to be prerequisites for the disease. The activated TLR4 promotes secretion of not only biglycan as described in the above-mentioned system but also cytokines that cause nephritis, such as CXCL13 and TNF α [10]. By adding back biglycan expression by pLIVE-mediated in vivo expression system in liver in the

biglycan knockout mice, it was clear that biglycan is not only necessary but also sufficient for the disease.

7.5.1.4 Decorin

Similar circumstances have been reported in decorin. Intraperitoneal injection of LPS at lethal dosages induced TNF α and IL-12p70 in the perivascular regions in the lungs with increased decorin expression (Fig. 7.2). The biological outcomes are inhibited by decorin knockout, which is restored by intravenous administration of decorin. This indicates that decorin is necessary and sufficient to LPS-induced lethality.

Interestingly, in a tumor xenograft model with lung cancer A549 cells infected by decorin adenovirus, TLR4 activation resulted in down-regulation of TGF β and IL-10, and PDCD4-mediated apoptosis of tumor cells. PDCD4 is a translational repressor of IL-10. It should be emphasized that the endogenous TLR4 ligand decorin is inhibitory for tumor progression (see below for S100A8 and SAA3 with an opposite effect) [8].

7.5.2 HMGB1

7.5.2.1 Biochemical Aspect

E. coli-expressed and purified HMGB1 (Fig. 7.3) with more than 90 % purity binds the TLR4/MD-2 complex with K_d 1.5 μ M as measured by surface plasmon resonance analysis (see Chap. 6). The binding was inhibited by anti-HMGB1 neutralizing antibody. Thus, HMGB1 is required for the binding even if it is not 100 % pure.

Precisely, HMGB1 changes its function in a manner dependent on the reduced status of the three conserved cysteine residues at 23, 45, and 106. The completely reduced form binds CXCL12 while the fully oxidized form lacks its TNF- α -producing activity. The disulfide HMGB1 that has a Cys23-Cys45 disulfide and the reduced Cys106 binds MD-2 with K_d of 12 nM but not TLR4 directly [11]. Screening HMGB1 peptide library gave P5779 located within the B box and capable of binding MD-2 with K_d of 0.65 μ M. Interestingly, P5779 had IC₅₀ of 5 μ g/ml for TNF α release induced by HMGB1 at 1 μ g/ml but failed to inhibit LPS-stimulated cytokine production implicating the different binding site in MD-2 from LPS.

Chemically synthesized 20-mer peptides within the B-box that was defined as the binding region of HMGB1 to the TLR4/MD-2 complex bind the receptor under K_d 22 μ M. A single amino acid substitution from Cys to Ser (C106S) abolished the binding.

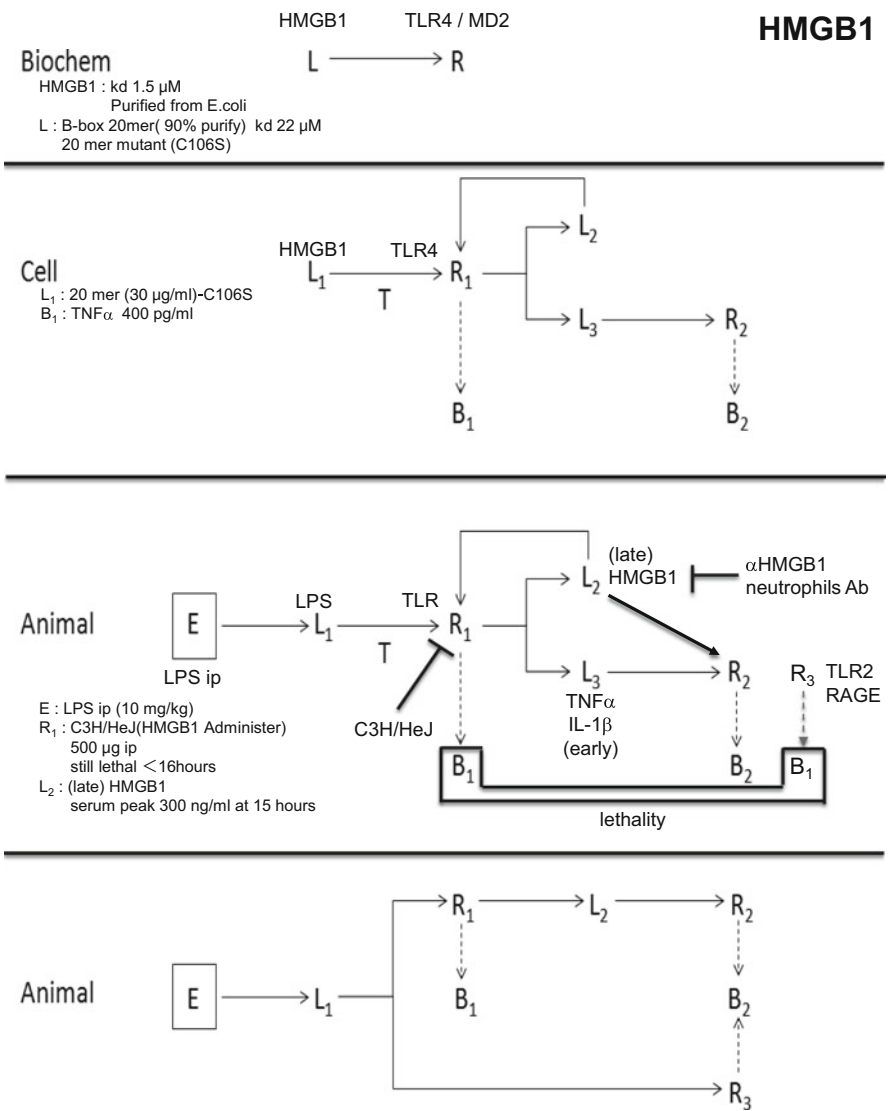


Fig. 7.3 Evidence for HMGB1 (See Fig. 7.1 legend for biglycan)

7.5.2.2 Cellular Aspect

The levels of contaminating LPS were <1 pg/μg of *E. coli*-purified HMGB1 proteins by LAL test. When peritoneal macrophages were stimulated by 1 μg/ml of the same HMGB1 preparation for 16 h, secretion of IL-6 at 3 ng/ml and TNFα at 500 pg/ml in the culture media was observed. Given that the contaminating LPS should be <1 pg/ml, its cytokine stimulatory activity is supposed to be negligible.

Interestingly, it was shown that the cytokine production was unaffected in macrophages deficient in either RAGE or TLR2, both of which were thought to be the second and third receptor for HMGB1. The 20-mer peptide within the B box at 30 µg/ml but not its mutant form C106S also was capable of inducing secretion of TNFα at 400 pg/ml 16 h after stimulation [12], which was totally abrogated in the background of TLR4 but not RAGE knockout [13]. The reader should be conscious of the experimental design in which theoretically LPS-free peptide ligands and receptors on the single population of responsive cells, both of them, are directly given loss-of-function mutations to prove that both HMGB1 and TLR4 are prerequisites for the cytokine production.

7.5.2.3 Animal Studies

Intraperitoneal injection of lethal dosages of LPS at 10 mg/kg in mice induced both TNFα and IL-1β production as an early response, which was followed by late serum elevation of HMGB1 up to 300 ng/ml peaked at 15 h. It is assumed that HMGB1 can amplify itself through TLR4 additionally producing the cytokine storm. This idea was demonstrated by the fact that anti-HMGB1 neutralizing antibody inhibited the lethality. In contrast to what is described above, in vitro macrophage stimulation experiments, RAGE and TLR2, may work in place of TLR4 to give similar consequences, because C3H/HeJ background failed to rescue the lethal phenotype.

7.5.3 *S100A8 and S100A9, and SAA3*

See Chap. 15 for details and demonstration as an endogenous TLR4 ligand.

7.6 Endogenous Antagonist Candidates

7.6.1 *OxPAPC (See Chap. 6)*

7.6.1.1 CTRP-3

C1q/TNF-related protein-3 (CTRP-3) is produced in monocytes and adipocytes and highly homologous to adiponectin [14]. Baculovirus-expressed and purified CTRP-3 inhibited LPS-stimulated CCL2 (MCP-1) release in differentiated 3T3-L1 adipocytes as well as IL-6 secretion in lauric acid-induced in human monocytes. The physical evidence by ELISA supported the antagonism.

7.6.2 *Gangliosides*

GM1 and GD1a inhibited production of cytokines including IL-12, TNF α , and IL-6 in human monocytes in which TLR2-8 were activated individually [15].

Combined Molecules of Lipid and Sugar

A ganglioside is composed of ceramide and oligosaccharide with N-acetylneuramic acid. Ceramide is an acylated sphingosine (long-unsaturated hydrocarbon chain amino alcohol), thus bearing two long hydrocarbon chains. Synthesis of globo-series of glycosphingolipid (GSL) starts with ceramide to which sugar residues, Glc, Gal, NAcGal, etc. are transferred one by one. When Glc and Gal (= lactose) are attached, it is called lactosyl-ceramide (Lac-Cer). The α 1,4-galactosyl transferase (A4galt) is one such transferases before globotetraosyl-ceramide (Gb4) is synthesized. A4galt-KO mice are susceptible to LPS-induced lethality with suppressed expression of TNF α , IL-6, and NOS2 [15] (Fig. 3.4 in Chap. 3 and Fig. 4.1 in Chap. 4).

7.6.3 *Globotetraosyl-Ceramide (Gb4)*

7.6.3.1 Biochemical Aspect

When pull-down experiments were performed with V5-tagged recombinant MD-2 and enzymatically prepared 3H-Gb4 after incubation of 3H-UDP-GalNAc and Gb4 precursors in the membrane, significant radioactivities were detected in the anti-V5 precipitates. Direct binding assay in native PAGE with purified Gb4 and the TLR4/MD-2 complex revealed Kd 238.1 μ M. In the same experimental condition under which LPS from *Salmonella minnesota* Re595 binds the TLR4/MD-2 complex with Kd 1 μ M, Gb4 inhibited the binding between LPS and the TLR4/MD-2 complex.

7.6.3.2 Cellular Aspect

LPS induced A4galt expression in primary culture mouse endothelial cells, which peaked at 9 h after stimulation. Exogenous Gb4 at 10 μ M, but not its precursor LacCer, blocked LPS-induced expression of NOS2, CXCL10 at 100 ng/ml in the endothelial cells.

7.6.3.3 Animal Studies

Intraperitoneal administration of Gb4 at 5 mg/kg counteracted against LPS-lethality with dramatic suppression of damages in the lungs, such as congestion and lymphocytic infiltration, as well as in the liver, including massive congestion, intralobular hemorrhage, and apoptosis. Those findings suggest that Gb4 is a pharmacologically noncompetitive complete antagonist of endogenous origin against LPS.

7.7 Some Interpretations of Published Reports

I never have an intention to criticize a paper already published in leading journals after elaborate works and reviews. I suppose that what you believe needs to be based on accurate and correct understanding of previous demonstration. Better evidence appears as science makes a progress. You cannot understand good papers correctly without correct interpretations of the evidence in the past. I introduce two papers (A *Nature* paper on influenza and Eritoran, citing a *Cell* paper on OxPAPC, see Chap. 6) where they found that two lung diseases are rescued by genetic background of TLR4 knockout and subsequently uncovered endogenous agonists for TLR4.

7.7.1 Lung Injury and TLR4

In acute lung injury caused by influenza virus, the lethal outcome can be abrogated by TLR4, but not TLR2 or CD14, knockout or the TLR4 signaling antagonist called eritoran (E5564), a tetra-acyl analogue of hexa-acyl lipid A portion of LPS (see Fig. 5.4 in Chap. 5) [16]. Crystal structure analysis revealed that eritoran binds deeply in the hydrophobic pocket of MD-2 but fails to dimerize TLR4. The therapeutic effect of eritoran is dependent on its binding to CD14 and subsequent transfer to MD-2.

A simple question rises. Does secondary infection by LPS-generating bacteria in the acutely injured lung actually kill the mice? The authors cited a paper at a first position in the reference published in *Cell* in which oxidized phospholipid as represented by OxPAPC, which was generated by oxidation by ROS produced in a Nox-2-dependent manner, was shown to be elevated in the injured lungs and claimed to function as an endogenous active mediator that stimulates TLR4 [17].

In the *Nature* paper, the eritoran effects on influenza mortality inversely correlated with the pulmonary levels of oxidized phospholipids as judged by matrix-assisted laser desorption ionization-imaging mass spectrometry (MALDI-IMS) and electrospray ionization-mass spectrometry. The authors claimed that they could find no bacteria or LPS in the lung homogenates from the influenza-infected lungs.

The evidence for TLR4 dependence and eritoran sensitivity was supplied in stimulation experiments of peritoneal macrophages by OxPAPC at 20 $\mu\text{g/ml}$ in which IL-6 mRNA levels were up-regulated by more than 20-fold. However, there have been accumulating publications by other groups showing that OxPAPC is an antagonist for LPS binding to any one of LBP, CD14, or MD-2 in human endothelial cells as monitored by NF κ B activation and expression of IL-8. OxPAPC alone failed to induce IL-6. Depending on the presence or absence of LPS, the functions of OxPAPC appear to differ from antagonistic to agonistic for TLR4 (see Note in Chap. 6).

7.7.2 Asthma and TLR4

Another biology that depends on the presence of TLR4 is the mouse model of asthma caused by intranasal inhalation of a fungal proteinase from *Aspergillus oryzae* (PAO) whose LPS content is 1.7×10^{-3} EU/ μg or ovalbumin administration [18]. Knockout of double but not either one of MyD88 and TRIF failed to induce asthma. The reliable manifestation of this airway disease model is airway constriction evaluated by respiratory system resistance and leukocyte, especially eosinophilic, infiltration in bronchoalveolar lavage fluid and mucin secretion. In the ovalbumin-induced asthma model, TLR4 also is necessary for the disease progression and production of IL-5 and IL-13, but interestingly not of IL-4 and IgE.

Then, what is the endogenous agonist for TLR4? Requirement of serum in PAO-mediated inhibition of fungal growth reminded the authors of endogenous TLR4 ligand candidates in serum, fibrinogen. The growth inhibition also took place when PAO was replaced by thrombin, indicating that fibrinogen cleavage is necessary for the antifungal effect. The fibrinogen cleavage product (FCP) directly could induce expression of the airway IL-5 and mucin Muc5ac in a TLR4-dependent manner. The classical Th2 cytokines include IL-4, IL-5, and IL-13 (see Chap. 2). The type 2 ILC in airways secretes both IL-5 and IL-13.

While IL-4 uses both type I and II IL-4 receptors, IL-13 uses type II. Type I consists of IL-4R α and common γ chains. Type II has IL-4R α and IL-13R α 1 chains. Given that airway resistance and mucin secretion are dependent on IL-13R α , TLR4 dependence of FCP-stimulated expression of IL-13R α 1 indicates that FCP not only activates but also sensitizes type 2 ILC for the Th2-derived cytokines to exert full phenotypes.

In all reagents for experiments in vitro and in vivo authors measured LPS levels by LAL, which turned out to be <2 pg/ml or 2 pg/mouse. As described in Chap. 5 self and non-self, PAR system activation by exogenous proteinases may cleave fibrinogen, which is then converted to functionally non-self peptides to stimulate TLR4 (see also Chap. 6 for the concept of self transformation to non-self).

Readers now should have a question. The idea that FCP is a TLR4 agonist lacks biochemical evidence to show that FCP and TLR4 (or MD-2) are both sufficient for direct binding.

7.8 An Idea of Sensitizer

HMGB1 binds not only TLR4 but also LPS and efficiently transfer LPS to CD14. In this situation, HMGB1 serves as a sensitizer for LPS, seemingly enhancing the LPS effects by increasing the efficiency of the true ligand LPS to bind the true receptor. The HMGB1 effect is mediated by the ligand LPS. In the case of DNA-TLR9 signaling, the sensitizing activity is mediated by RAGE, a receptor for the DNA (see Chap. 6).

Studies of HSP family of proteins as endogenous TLR4 ligands have been hampered by their strong affinity to LPS. The battle against contamination was resolved by modification of purification procedures, which has given a negative result as TLR4 ligands. However, one member Gp96, also called GRP94 (see ref. [19] for the nomenclature guideline of HSP family of proteins), was found to be tightly associated with LPS during Gp96 purification. However, when separated from each other, stimulation of bone marrow-derived dendritic cells by LPS at 250 pg/ml, which gave no response in their experiments as monitored by expression of IL-6 and IL-12p40, was dramatically augmented when LPS had been preincubated with Gp96 at 25 µg/ml for 1 h at 37 °C before application, whereas Gp96 alone failed to do so [19]. While Gp96 is thought to chaperone TLR9, macrophage-specific deletion of Gp96 resulted in unresponsiveness to ligands for TLR9 and TLR4, both of which could bind hyperglycosylated Gp96. Therefore, it is likely that another sensitizer Gp96 binds both the true ligand LPS and true receptor TLR4 and that Gp96 is necessary and sufficient for TLR4 activation by LPS.

7.9 Concluding Remarks

Logical and technical supports with careful examinations are required to show the presence of a genuine TLR4 ligand of endogenous origin. Its biological significance should be challenged without discrepancy at both cellular and animal levels.

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Chapter 8

Autoinflammatory Disorders

We have discussed the actual presence of the endogenous ligands for TLR. In this chapter, I will describe their potential involvement in human diseases. Given that metastasis is a clinical name or name of a disease, I believe that any research irrelevant to or in disagreement to clinical information is of low value. There is no doubt that fever is one of the fundamental symptoms of ambiguously defined “inflammatory disorders” in clinical settings. For example, if clinical manifestations of a patient involve fever of unknown origin (FUO), being febrile $>38.3^{\circ}\text{C}$ for more than 3 weeks with failure to reach diagnosis, medical doctors usually think of (1) infectious, (2) neoplastic, and (3) noninfectious inflammatory diseases [1]. The third group mainly includes autoimmune disorders, such as systemic lupus erythematosus (SLE) (see Chap. 4), which affect connective tissues. Patients with autoimmune disorders almost always have autoantibodies, pathologically directed against their own cells or tissue components as seen with Guillain-Barré syndrome (see Chap. 4). However, their etiology is not well defined.

I will describe autoinflammatory diseases (AID) that belong to the third category. Their critical feature is the lack of infection, indicating that their cause is deterioration of self (auto- meaning self) in a strict sense. Non-self generated by mislocalization of self as discussed in Chap. 6 is included. The list of the disease is continuously expanding with the help of extensive genomic analysis, which has actually uncovered their respective genetic alteration(s), supported subsequently by experimental results. Therefore, it is a little too early to define the disease entity before the establishment of a completely understandable profile. However, I like the classification shown in Table 8.1, which is a modified version of what was proposed by Dan Kastner at NIH, because it seems to comprise any related diseases that might join in the future [2]. Some of them are really rare, but the important point is that they are indeed valuable simply because the background is human not other animals. I do not have an intention to explain all of these diseases. Instead, I will close up some useful ones by which to describe molecular pathogenesis. We need the information in the coming Parts III and IV.

Table 8.1 AID classification

	Disease	Gene locus	Protein	Inheritance
I	IL-1 β activation disorders (inflammasopathies)			
	FMF	MEFV 16p13.3	Pyrin	AR
	MKD = hyper IgD syndrome (HIDS)	MVK 12q2A	Movalonate kinase	AD
	FCAS, MWS, NOMID	NLRP3 1q44	Cryopyrin	AD
	PAPAs	PSTPIP1	CD2 anti-gen-binding	AD
		15q12.1–13	Protein 1	
	MS	LPIN2 18p4434	Lipin2	AR
	DIRA	IL1RN2q14	Interleukin-1 Receptor antagonist	AR
II	NFkB activation disorders CD is a complex form of III			
	NLRP12AD = FCAS2 (Guadeloupe periodic fever)	NLRP12 19q13	Monarch-1	AD
	BS	NOD2 (CARD 13) 16q12.1–13	NOD2	AD
III	Protein folding disorders			
	TRAPS	TNFRSF1A	Tumor necrosis	AD
		12p13	Factor receptor 1	
	Nakajo-Nishimura syndrome	PSMB8	6p21	
IV	Complement disorders atypical HUS (GFH, Iq32, MCP1q32, CFI4q25)			
V	Cytokine signaling disorders cherubism, SH3BP2 4p16			
VI	Macrophage activation disorders fHLH			
	PRF1 (perfolin) 10q22, UNC13D 17q21, STXII 6q24			
	Acquired form of fHLH: still disease			

The affected genetic locus with corresponding proteins and inheritance mode, i.e., AR or AD are shown. For example, a PSTPIP1 mutation was documented in Ref. [36]. Modified from Ref. [2] *fHLH* familial hemophagocytic lymphohistiocytosis, *TRAPS* TNF-receptor–associated periodic syndrome, *FMF* familial Mediterranean fever, *AR* autosomal recessive, *AD* autosomal dominant, *CD* Crohn’s disease

They share common clinical manifestations, including periodic fever, arthritis, skin rash, and lack of autoantibody found in autoimmune disorders. However, monogenetic alterations of inheritance have been demonstrated to contribute their

pathogenesis. Genetically diverse but clinically similar characteristics are analogous to cancer. Given that they are essentially disorders of deregulated interleukin (IL)-1 β and TNF α that are significantly influenced by TLR signaling, I first show how expression and function of those two cytokines are regulated. Second, I will describe some of the autoinflammatory diseases in a mechanistic fashion and lastly discuss the possible involvement of endogenous TLR ligands in them.

8.1 Fever as a Historical Golden Marker of Inflammation

A variety of components derived from microorganisms induce fever in mammals as represented by LPS, which has been extensively studied due to its clinical importance involved in septic shock. Those exogenous pyrogens stimulate the expression of endogenous mediators that eventually stimulate the thermoregulatory neurons in the preoptical anterior hypothalamus. The distal or peripheral mediators at least include IL-1 α and IL-1 β (hereafter referred to as IL-1 unless otherwise indicated specifically as IL-1 α etc.), TNF α , and the proximal or central mediators in the brain are IL-6, PGE₂, IL-1, and TNF α . The fever eventually goes down by the activation of the hypothalamus-pituitary-adrenal gland axis with a final antagonistic product glucocorticoid.

Remember the necessary and sufficient logic discussed in Fig. 4.3 in Chap. 4. The intra-cerebro-ventricular (ICV) injection of PGE₂ induces fever and intravenously injection of LPS showed a febrile consequences in either COX-2 or EP3 knockout mice [3, 4]. Thus, PGE₂ is an evident proximal mediator for fever by binding hypothalamic EP3. The LPS in the peripheral tissues induces expression of both IL-1 and TNF α , which are released into circulation but can hardly penetrate through the blood-brain barrier. However, their receptors are present there, and both cytokines can pass the baton to the brain where both IL-1 and TNF α are expressed [5]. They then stimulate the expression of both COX2 and IL-6, which reciprocally activate the expression of the other. The COX2 product PGE₂, when injected ICV, exerts only transient fever even in the background of IL-6 knockout [6]. Intraperitoneal TNF α can induce COX2 expression in the brain in the IL-6 knockout mice but with no fever. LPS can still induce fever in IL-1R1 knockout mice. Therefore, it is assumed that COX2-mediated IL-6 expression may play a central role in the brain for fever.

With regard to 2-atom molecules, such as nitric oxide (NO) and superoxide, one can hardly demonstrate that they are sufficient for a certain biology because of their gaseous nature for administration. However, in mice deficient in either inducible NO synthetase (iNOS or NOS2) or neuronal NOS (nNOS or NOS1), but not endothelial NOS (eNOS or NOS3), systemic application of LPS resulted in no fever, indicating that they are at least necessary for LPS-induced fever [7]. However, both iNOS and nNOS are not required for fever induced in the sterile inflammation model with turpentine oil that is injected to subcutaneous tissues in which proximal mediators are presumed to play a significant role.

Studies in human volunteers showed that 1–10 mg/kg of IL-1ra failed to suppress febrile responses by a bolus injection of LPS at 3 ng/kg but suppressed elevation of leukocyte numbers in the peripheral blood [8]. The Fc portion-tagged TNFRp75 subunit could hardly diminish LPS-induced febrile responses, indicating that TLR4 triggering in brain endothelial cells could cause fever in a manner independent of IL-1 or TNF α .

8.2 IL-1 β in the IL-1 Family

It is worth noting that historical efforts to identify endogenous mediators of microbial pyrogen have led to the discovery of what was called “endogenous pyrogens” at that time. The fact that they have pleiotropic effects causing more than fever, including increased mobilization of neutrophils from bone marrow, elevation of serum amyloid A (SAA1 and 2) from liver, activation of sympathetic nervous system, activation of macrophages to produce lymphocyte-activating factor (thus named *interleukin*), renamed the endogenous pyrogen IL-1 before isolation of their cDNAs. The recently expanded IL-1 family members were assigned an individual interleukin designation (Fig. 8.1) [9].

IL-1 α and IL-1 β share only 20–30 % homology on amino acid levels but show a high structural similarity [10]. The intravenously administered radiolabeled human IL-1 β showed a short half-life of 5–10 min and mainly eliminated in urine. IL-18 by itself does not serve as an endogenous pyrogen, possibly because it cannot induce COX2 but can induce expression of three pyrogenic cytokines IL-1 β , IL-6, and TNF α .

There are several unique features in this family of proteins. IL-1 α , IL-1 β , and IL-37 lack signal sequence responsible for the conventional ER-Golgi routing and secretion into the extracellular space. On necrotic cell death, they can be passively released out of the cells. IL-1 α even binds nuclear DNA. IL-1 α , IL-37, and IL-33 translocate into the nucleus after cleavage by activated caspase-1 and suppress LPS-stimulated transcription. There is a group of people who refer to this nucleus-oriented, namely movement of molecules not toward extracellular space <paracrine> or into circulation <endocrine> but toward itself as <intracrine,> However, IL-18 and the most extensively studied IL-1 β are actively secreted into extracellular space. This is executed by a special scaffold complex consisting of NLRP3 (nucleotide-binding domain and leucine rich repeat containing family, pyrin domain containing 3), ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain [CARD]), and pro-caspase-1. NLRP3 was previously called cryopyrin whose mutation was found in familial cold autoinflammatory syndrome. NLRP3 contains a PAMP or DAPM-recognizing C-terminal leucine rich repeat as found in other pattern recognition receptor, such as TLR, a central nucleotide-binding domain (or NACHT domain, named after NAIP, CIITA, HET-E, and TP1), and N-terminal pyrin domain (Fig. 8.2). ASC, a bipartite

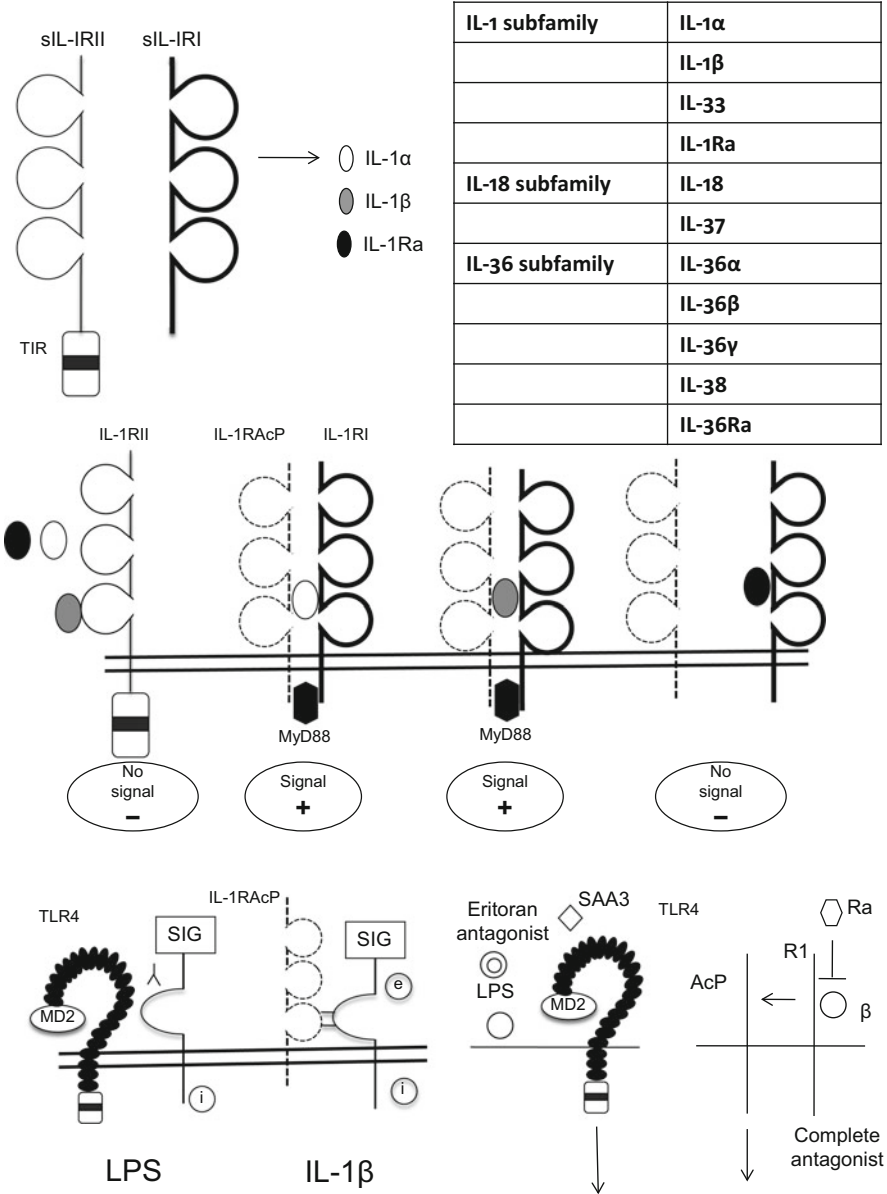
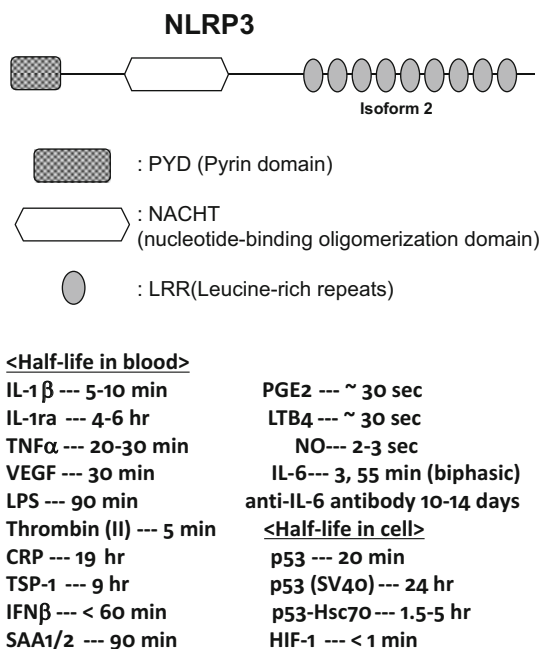


Fig. 8.1 IL-1 family and its receptor. Ligand-dependent receptor dimerization is essential for signaling. Shown below is SIG affecting both TLR4 and IL-1R signaling [37]. Accessory factor MD-2 for TLR4 binds LPS, its antagonist Eritoran and SAA3 for competition

Fig. 8.2 NLRP3 structure.

There are at least six isoforms in the LRR domain due to alternative splicing. The isoform 2 of the canonical sequence is shown. NLRP3 is activated by uric acid [38], cholesterol [39], calcium oxalate crystal [40], SAA [15], and oxidized mitochondrial DNA [41]. The plasma half-life of IL-1 β that is released from NLRP3 inflammasome is short, i.e., 5–10 min [42]. For reference, those of other cytokines are shown, including LPS [43], IFN β [44], IL6 [45], LTB4 [46], and those of intracellular proteins within the cells such as HIF-1 [47] also are shown



adaptor, connects NLRP3 and pro-caspase-1 via homotypic interactions of its pyrin and CARD domain, respectively. The complex is called inflammasome.

A variety of stimuli can activate the inflammasome, including ATP, which activates P2X7 receptor, various crystals, bacteria, and so forth [11]. This poses similarities to the recognition of “danger” in TLR described in Part I.

However, different from the crystal structure level of understanding of LPS directly binding MD-2 with subsequent dimerization and activation of TLR4, activation mechanisms of NLRP3 still await precise elucidation. An assumed mode of activation is as follows. An intramolecular binding between the NACHT and LRR domains gives an autoinhibited conformation. Stimuli open up the structure allowing oligomerization through the NACHT domains. The pyrin domain contains Cys responsible for disulfide bond formation before oxidation by ROS, suggesting a redox-sensitive nature of NLRP3.

Then, what is the sensor molecule that leads to the activation of inflammasome in a mechanistic manner? LPS can up-regulate transcription of IL-1 β mRNAs that are translated to IL-1 β precursor proteins ready to be cleaved by caspase-1 to its mature form. In LPS-induced activation, ATP can provide a second signal for the activation by binding to its receptor P2X7 allowing pore formation by pannexin-1 hemi-channel and inducing K efflux. The former permits entry of extracellular NLRP3 agonists for direct access in the cytoplasm. Then, what is the triggering mechanism of ATP release into extracellular space? Cell injury could be one reason. Hyaluronan from damaged cells also activate inflammasome. In a mouse skin sterile inflammation model using beads or liquid nitrogen in which hyaluronan

serves as DAMP, IL-1 β protein is up-regulated in those tissues in a manner dependent on NLRP3 or TLR4 [12]. When peritoneal macrophages are stimulated in vitro, hyaluronan-stimulated transcription of IL-1 β mRNAs and released amount of IL-1 β are suppressed in TLR4 knockout. Because hyaluronan receptor CD44 not only is internalized allowing intracellular interaction with NLRP3 but also associates with TLR4, hyaluronan alone causes effects equivalent to the combined outcomes of LPS and ATP.

Different from DAMPs derived from injured tissues, more natural proinflammatory mediators are proposed, including SAA1/2 and cathelicidin-derived leucine leucine-37 (LL-37) peptide, both of which are claimed to stimulate directly P2X7 receptor with pore formation [13, 14]. When human recombinant SAA1/2 proteins from *Escherichia coli* with LPS contamination claimed to be <3 ng/ml were subjected to stimulation experiments with human peripheral monocytes, they induced IL-1 β up-regulation in both mRNA and protein levels, indicating inflammasome activation [15].

8.3 Inhibitors of NLRP3

8.3.1 Nitric Oxide

NLRP3 is the founder of the concept of a larger family called NLR (see Table 2.1 in Chap. 2 for PRR) additionally, including other NLRP family members, NOD, IPAF, etc. NLRP3 suppressors also are proposed. Activation of inflammasome by both LPS and ATP is suppressed by NO donors, such as S-nitroso-N-acetylpenicillamine (SNAP) [16]. When duration of priming by LPS is extended to 24 h, ATP-induced IL-1 β secretion is eliminated. This can be reversed by iNOS knockout. Given that LPS up-regulates expression of iNOS generating large amounts of NO, NO may give negative feedback to the activation.

8.3.2 Presence of Endogenous IL-1 β Inhibitors

The receptors of IL-1 family members are heterodimers of one ligand-binding chain with which coreceptor chain associates to transmit intracellular signaling. Both IL-1 can bind both IL-1R1 and IL-1R2. However, lack of cytoplasmic sequences for signaling converts the IL-1R2 a decoy receptor. Another mechanism of negative regulation in IL-1 signaling is found at the ligand level. Of note, the endogenous pyrogen IL-1 has its own endogenous inhibitor called IL-1R antagonist (IL-1Ra). IL-1Ra is induced by IL-1 itself, which gives a negative feedback loop. IL-1Ra serves as a complete antagonist by forming a complex IL-1Ra-IL-1R1 that cannot associate with IL-1RAcP due to its internal structure difference as judged by their

crystal structures. IL-36 has a similar inhibitor IL-36Ra. The third inhibitor is SIGGIR that can bind either TLR4 or IL-1RAcP to inhibit IL-1 and LPS signaling, respectively. Therefore, IL-1 β is regulated at the levels of transcription, its receptor, and processing.

8.4 TNF α in the Middle of Large TNF and TNFR Families

Gratia and Linz found in 1931 that LPS induced hemorrhagic necrosis of transplanted liposarcoma in guinea pigs with *E. coli* culture filtrates. It was understood as Schwartzman reaction that can damage endothelial cells. The purified substance from LPS-stimulated cells, but not LPS by itself, causes cell death in vitro. Thus named tumor necrosis factor was found to be identical to the molecule that causes cachexia in 1985 [17, 18]. This is the discovery of TNF, but its effect on tumor is discussed in Chap. 16.

I will not describe all the members of the two families but focus on TNF α and TNF receptor 1 (TNFR1, CD120a, p55) (Fig. 8.3). Although TNF has a second receptor TNFR2 or p75, knockout mice studies revealed that TNFR1 predominates over the TNFR2 in the proinflammatory activities. A variety of types of cells produce TNFRs but its ligand TNF α is synthesized in immune cells and keratinocytes. Both TNF α and TNFR1 belong to a huge family of transmembrane protein and are unique in that they can be released from the cell membrane by proteolytic cleavage. The ectodomain shedding of TNF α is achieved by TNF- α -converting enzyme (TACE) that is alternatively named ADAM17, which is further regulated negatively by TIMP-3 that is under the transcriptional control by c-Jun and JunB. ADAM17 also can shed TNFR1 and TNFR2. TNFR release is observed by a variety of stimuli, including TNF α itself, LPS, and IL-10.

8.5 Autoinflammatory Diseases

Next, I will discuss human diseases. Familial Mediterranean fever (FMF) and deficiency of IL-1-receptor antagonist (DIRA) in the group IL-1 β activation disorders, TNF-receptor-associated periodic syndrome (TRAPS) and Nakajo-Nishimura syndrome (NNS) in protein folding disorders, and one experimentally engineered animal disease of A20 deficiency. FMF had drawn clinical attentions for a long time until its pathological mechanism was uncovered by knockout mice studies of the discovered gene. Conversely, the gene for DIRA, IL-1Ra, was well-known before the disease entity was proposed with a group of patients. In both cases, it should be stressed that animal models cannot necessarily recapitulate the clinical signs and symptoms. However, we need animal models by which to translate what is shown to the possible strategy for treatment.

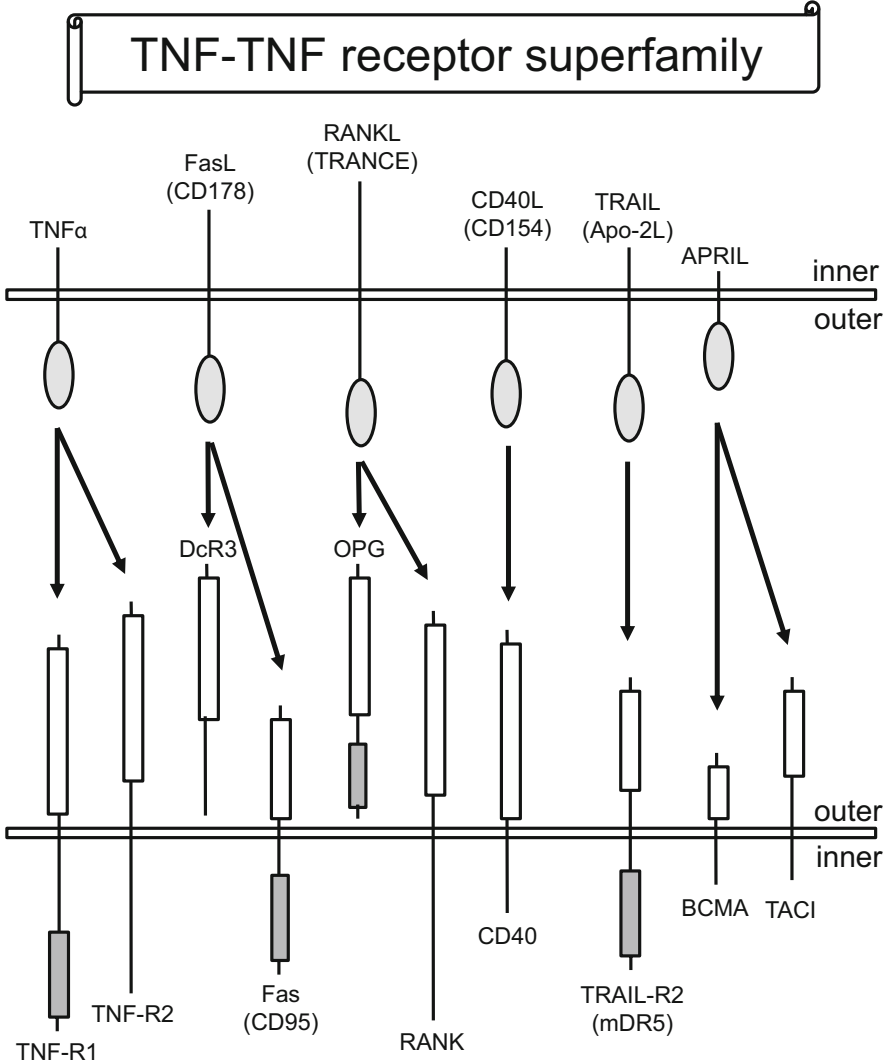


Fig. 8.3 TNF and TNFR family. Six members of the whole family. Both TNF and TNFR members are actually of trimeric structure but depicted as a single bar. For example, TRAIL induces activation of caspases by binding to cell death receptor DR5

8.5.1 Familial Mediterranean Fever

As mentioned earlier, fever is not necessarily a symptom of microbial invasion. The longest-observed disorder was described in 1945 as “benign paroxysmal peritonitis.” Recurrent and self-limited (not communicable to others or no apparent infection) episodes of fever up to 40 °C with symptoms of serositis, such as abdominal

pain due to peritonitis, were recessively inherited in a group of young people of eastern Mediterranean ancestry and now called FMF [19]. Positional cloning identified the gene pyrin. Most FMF patients carry missense mutations in the C-terminal half, truncation of which in knock-in mice demonstrated exaggerated LPS-induced hypothermia with increased IL-1 β production in LPS-stimulated resident peritoneal macrophages derived from those mice [20]. Nonmutated full-length pyrin binds and sequesters ASC, resulting in blocking the interaction between procaspase-1 and ASC, which eventually leads to an inability to activate IL-1 β . One hypothesis is that the reduced inhibition of IL-1 β processing by mutant pyrins may be the cause of FMF.

8.5.2 Deficiency of IL-1–Receptor Antagonist

The disorder is recessively inherited and heterozygous carriers are asymptomatic. In 2009, nine children of homozygous mutations of IL-1Ra were reported [21]. The genetically altered gene products failed to be secreted to exert the inhibitory function of IL-1Ra. Pustular rash with prominent neutrophil infiltration with high expression of IL-17 in both epidermis and dermis, sterile but purulent osteomyelitis, joint swelling, elevated erythrocyte sedimentation rate, and marked C-reactive protein levels are observed. The elevated cytokines in the patient monocytes stimulated by IL-1 β include IL-1 α , MIP-1 α , TNF α , IL-8, and IL-6. The dermatological and bone lesions ameliorated by treatment with anakinra (IL-1ra is a recombinant version of natural IL-1Ra with a half-life 4–6 h), discontinuation of which resulted in relapse within 36 h. However, no patient had fever. The IL-1 β levels were not elevated (Ivona Aksentjevich, personal communication). IL-1Ra knockout mice showed polyarthritis but autoantibodies against double strand DNAs were present, suggesting tissue-specific inflammation of autoimmune nature.

8.5.3 TNF-Receptor–Associated Periodic Syndrome

This disease is caused by single nucleotide missense mutations in the extracellular domain of the TNFR1 responsible for ligand binding and homo-trimerization (Fig. 5.3 in Chap. 5). The febrile episode lasts more than a week to even 1 month, longer than FMF, suggesting a persistent presence of inflammation. Low serum levels of soluble TNFR, elevated SAA1/2, and IL-6 are observed. The disease is inherited in an autosomal dominant manner. In both patients and knock-in mice analyses, mutant TNFR1 proteins accumulate in the endoplasmic reticulum (ER) capable of inducing unfolded protein response that directly activates NF κ B and never function as a cell surface receptor for TNF [22]. Heterozygous mutant mice are paradoxically sensitive to LPS for shock, but homozygous ones are equivalent to TNFR perfect knockout and resistant to LPS. In the former activation

of both p38 and JNK is observed, which may explain the fact that TRAPS patients are hypersusceptible for LPS at low dosages.

8.5.4 *Nakajo-Nishimura Syndrome*

This disease was originally documented in Japan and segregates in an autosomal recessive manner [23]. Homozygous mutations in the b5i subunit are uncovered. In addition to the proteolytically active b1, b2, and b5 subunits in the usual 20S proteasome, IFN γ -induced b1i, b2i, and b5i are integrated in immune cells, such as macrophages to form a special proteasome called immune-proteasome. Mutations are found in b5i. Although p65/p50 heterodimer levels and JNK activation are not affected, increased activation of p38 is specifically observed in the nucleus of patient cells. Sustained inflammation in this disease may be due to IL-6 overproduction as in the case of TRAPS.

In clinical settings, humoral biomarkers are, but not always, IL-1, IL-6, TNF α , and those induced by them, which include CRP, SAA1, SAA2, fibrinogen, S100A8, S100A9, S100A12, and so forth.

8.5.5 *Kawasaki Disease from Japan*

Kawasaki disease is an ANA-negative vasculitis of median size. A single nucleotide polymorphism (SNP) of the inositol 1,4,5-triphosphate (IP3) kinase C (ITPKC) was shown to confer sensitivity to Kawasaki disease and its major complication: coronary arterial disease [24]. The ability of IP3 to release Ca $^{2+}$ from endoplasmic reticulum or sarcoplasmic reticulum is inhibited by phosphorylation by ITPKC to IP4. Therefore, the SNP, which results in reduced splicing efficiency of the ITPKC mRNA, de-inhibits the Ca $^{2+}$ release from the storage. This causes activation of T cells and smooth muscle cells through the aberrantly activated calcineurin/NFAT pathway. In fact, it was reported that calcineurin inhibitors (CI), such as cyclosporine and tacrolimus, gave resolution of inflammation, such as fever in ten Kawasaki disease patients who were resistant to intravenous immunoglobulin therapy. It is expected that a combination of high-dose immunoglobulin, aspirin, and cyclosporine A can reduce the risk of coronary artery complications [25].

CI FK506 was reported to behave like LPS to produce TNF α in peritoneal macrophages, which was reduced in KO of TLR2, TLR4, MyD88, or TRIF, and even to augment the LPS effect at low concentration of 10 ng/ml but not 100 ng/ml. Mechanistically, calcineurin could inhibit NF κ B activation elicited by TLR4, but not TNF α R, by interacting with MyD88, TRIF, and TLR4 as assessed by co-immunoprecipitation. Generally, however, aggravation of inflammation is not documented under CI treatment in patients. I assume that TLR4 activation by

de-inhibition is more easily subjected to negative feedback than that by direct stimulation [26].

8.5.6 Rheumatoid Arthritis

Whereas a large genome wide association study (GWAS) with 29,880 rheumatoid arthritis (RA) cases and 73,758 controls analyzing ten million single-nucleotide polymorphisms (SNPs) recently predicted 101 genetic-risk foci, of which 42 are novel, practical targeting IL-6 and TNF α in clinics has been successful [27]. In addition, the study proposed repurposing the drugs that were originally developed for other diseases. For example, both anti-CD20 antibody rituximab and anti-CDK4 inhibitor flavopiridol target human malignant lymphoma. There are two important points to consider. First, given that rituximab is already in clinical practice, the underpinnings of RA and cancer should have some elements in common that are crucial for disease progression. Second, we need to look for the most effective drugs among the multigenetic alterations.

8.5.7 Chediak-Higashi Syndrome

This is an autosomal recessive disorder with loss-of-function mutations in lysosomal trafficking regulator (LYST) gene and represents a hyperinflammatory disease called hemophagocytic lymphohistiocytosis (HLH) [28]. Typical inclusion bodies are found in Chediak-Higashi syndrome (CHS) granulocytes. Basically, HLH has defects in perforin or proteins engaged in the transport of perforin-containing lysosomes as in CHS. In addition to NK cells, CTL functions are impaired. Lack of perforin function in infection, for example, induces excessive macrophage activation as observed in phagocytosis of normal host cells. The beige mice carry a mutation in the LYST gene (see Chap. 9).

8.5.8 Experimental A20 Deficiency: Antagonism against NF κ B Signaling

Different from the monogenic (monogenetic) diseases exemplified above, most well-known inflammatory diseases, such as RA and inflammatory bowel disease (IBD) are caused by multiple genetic alterations. For example, some patients with Crohn's disease have mutations in the LRR region of NOD2. However, it is just one of the susceptibility alleles that predispose to the disease. Mice deficient in NOS2 fails to develop IBD. By contrast, dominantly inherited NOD2 mutations in the

NACHT domain are found in Blau syndrome, which affects uvea, joint, and skin but not intestinal tracts.

Conversely, SNPs in the gene called A20 (alternatively called TNFAIP3; TNF- α -induced protein 3) are associated with susceptibility to a subset of inflammatory disease, including Crohn's disease, psoriasis, and RA [29–31]. A20 deubiquitinates key NF κ B-signaling molecules, such as RIP1 and TRAF6, thereby negatively regulating stimuli from IL-1R1, TNFR, TLR, and NOD2 (Fig. 5.3 in Chap. 5).

IL-6-neutralizing antibodies and TLR4-neutralizing *Rhodobacter sphaeroides* LPS, but not TNFR genetic deficiency, ameliorate RA-like phenotypes, such as bone and cartilage destruction caused in A20-deficient mice, indicating an essential contribution of TLR4-dependent signaling pathways in the mice.

8.6 Organotropism in Inflammatory Diseases

Affected organs and tissues include skin, joint, eye, intestinal tract, and so forth. When TNF α is overexpressed in the skin with the keratin-14 promoter, not only dermatological deteriorations in the dermis, including fat loss, increased fibroblast number, cell debris with heavy infiltration of neutrophils and macrophages, but also cachexia with elevated serum TNF α were observed showing that TNF α generated in the epidermis was released into circulation to cause, directly or indirectly, pleiotropic effects via TNFR expressed in most cells. Therefore, it is likely that wherever the TNF α is expressed, the affected tissues should have the receptors for TNF α and/or sequentially expressed downstream cytokines.

In FMF, CAPS, and TRAPS, elevated serum SAA1 and SAA2 are amyloidogenic and renal damages by renal amyloidosis take place rather efficiently [32]. Transformation of protein structure after secretion also causes further effects.

8.7 Lessons from Patients: Relevance to TLR and its Endogenous Ligands

Although mouse engineering can recapitulate aforementioned human genetic disorders of inflammation, progress in clinical medicine can provide us with valuable information from clinical settings that is sometimes in disagreement with that from animal models. In CAPS, increased serum levels of IL-1 β reflect that its up-regulation and anti-IL-1 β monoclonal antibody (canakinumab) therapy, but not that by TNF inhibitors, is effective and serum levels of the S100A8/S100A9 heterodimers serve as a good biomarker to monitor progression and remission of the disease. This raises a fundamental question of whether S100A8 and/or S100A9 are real effectors of this IL-1 β activation disorder [33]. In PAPA, the S100A8/S100A9 heterodimer also is elevated in the patient serum.

The linkage between IL-1 β and S100A8/S100A9 is well studied in Still's disease, alternatively called systemic-onset juvenile idiopathic arthritis (SOJIA) [34, 35]. It is thought to be an acquired form of familial hemophagocytic lymphohistiocytosis (fHLH). Although SOJIA lacks specific immunological biomarkers, such as rheumatoid factor and ANA, roughly 20 % of those patients were reported to be heterozygous for missense mutations in perforin essential for NK cells, but they are not monogenically inherited. Although SOJIA patients do not present high concentrations of IL-1 β , presumably due to its short half-life of <10 min, those of IL-18, another NLRP3-dependent IL-1 family member, are elevated. The elevated serum levels of S100A8/S100A9 heterodimer in SOJIA patients were dramatically decreased in response to the treatment by IL-1ra (anakinra). S100A9 proteins purified from mammalian cells can induce IL-1 β secretion in human monocytes with concomitant increase of its mRNAs by more than 200-fold. There is a report that S100A8 and S100A9, although purified from *E. coli* and with LPS levels claimed to be <1 pg/mg of protein, individually activated NLRP3 and NF κ B in a ROS-dependent manner and thereby inducing IL-1 β as well as IL-18 (only by S100A9), TNF α , and CCL2. Conversely, IL-1 β , GM-CSF, or LPS individually stimulate secretion of the S100A8/S100A9 heterodimers in human monocytes. Thus, it is likely that there exists an amplifying mechanism in monocytes for both IL-1 β and S100A8/S100A9 heterodimer in SOJIA. Which comes first—this is an egg and chicken question.

In addition to autoinflammatory diseases, S100A8 and S100A9 are up-regulated in infection and autoimmune diseases, including SLE, ANCA-associated vasculitis, psoriatic arthritis, adult-onset Still's disease, Kawasaki disease, dermatomyositis, and rheumatoid arthritis.

Colchicine has been long utilized as an effective drug in the acute attack as well as the prophylaxis of gout (see Chap. 5). It is not effective for acute attack of FMF but is in its prophylaxis and for the prevention of amyloidosis. Colchicine is an alkaloid derived from the seeds of *Colchicum autumnale* and binds its high-affinity endogenous receptor β -tubulin. Microtubules form cellular cytoskeleton that plays an essential role particularly in mitosis and cell migration. The above-mentioned effects of colchicine in gout and FMF have been believed to be due to the inhibition of chemokine-driven cell migration in the body. Recent cDNA microarray analyses in human endothelial cells added more information on the mechanisms of its action. It suppressed the expression of proinflammatory genes, including caspase-1, NOS3, and RhoC. Although it may not be a direct effect on transcription machinery, the microtubule disturbance should certainly change many cellular activities, including gene expression.

8.8 Linkage between Autoimmune Disease and Autoinflammatory Disease

The difference between autoimmune disease and autoinflammatory syndrome is not defined accurately even in clinical settings. In basic research, autoantibody against dsDNA is present in IL-1Ra knockout mice with arthritis as I mentioned earlier. Also, experiments with Unc93b13d lupus-prone mice in which nucleic acid sensing TLR signaling is missing, establishment of the autoantibodies against not only nuclear DNA but also nonnuclear antigens, such as RBC (anti-RBC antibody) and Fc portion of immunoglobulin (anti-Fc antibody = rheumatoid factor), was found to require the TLR signaling. While presence of a high titer of autoantibodies represents the autoimmune nature, spontaneous remission and genetic mutations of inflammatory cytokines and their regulators show the autoinflammatory property. The triggering mechanisms in the former may be constant existence of tissue-damaging autoantibodies and those of the latter could be extrinsic factors, such as microbes that exert inflammatory reactions in genetically deregulated background of cytokines. However, depending on what sort of elements dominates in the life-threatening tissue injury, target molecules or cells should be challenged.

8.9 Concluding Remarks

Genetic alterations have been uncovered in autoinflammatory diseases. Most of them are related to innate immune genes, such as IL-1 β , TNF, NLRP3, and their signaling molecules. Importantly, those systems are closely engaged with TLR signaling pathways of both extrinsic and endogenous ligands.

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Part III

Cancer

Chapter 9

Cancer in General

9.1 Overview

I define the term “cancer” as a combined concept for both tumor and leukemia. Tumor represents mass or solid tumor in a strict sense and leukemia seldom forms mass. However, in order for tumor to achieve hematogenous metastasis, the tumor cells need to make an entry into circulation as leukemic cells usually do. Some researchers call circulating tumor cells (CTC) “tumor cells in leukemic phase.” Both malignancy and neoplasm are synonymous with cancer.

Cancer research started with tumors of nonhuman origin, including rodent, chicken, and cat. The transferrable nature of those tumors is dependent on retrovirus oncogenes that can transform sensitive fibroblasts or hematopoietic cells in vitro and in vivo. Isolation of their cellular counterparts revealed that they are mostly growth factor receptors with tyrosine kinase activity. Discovery of activating mutations of cellular oncogenes is exemplified in v-ABL of Abelson murine leukemia virus and BCR-ABL found in human chronic myeloid leukemia (Table 9.1). In addition to those gain-of-function mutations, loss-of-function alterations of genes, such as those of p53, were found in many human cancers, and they are called tumor suppressors. Those nonphysiologically generated abnormal proteins are subjected to degradation and not allowed to survive within the cells. However, the mutated proteins escape the cellular protein quality control program and are stabilized by, e.g., heat shock proteins. Both growth-promoting and -suppressing activities are explained in the concept of signal transduction and eventually reflected in the cell-cycle progression and arrest. The real-player molecules involved in cell cycle are located in the nucleus, because mitosis always needs to accompany replication of DNA in which they are closely engaged. Under physiological conditions, cell proliferation is tightly controlled by telomerase activity. Conversely, uncontrolled mitotic activity or immortalization is associated with deregulated telomerase activity in tumor cells. Not only cell proliferation but also cell death is deregulated in tumor cells. Functional alterations of Bcl-2 with

Table 9.1 Historical discovery and ideas. We should remember the atomic bombings

Time table	
ca 460–370 BC	Inflammation (Hippocrates)
1786	Metastatic disease (John Hunter)
1865	Milieu interior (Claude Bernard)
1889	Seeds and soil (Stephen Paget)
1911	Rous sarcoma
1913	Partial resection and metastasis
1927	X-ray causes genetic mutations in flies
1943	Yoshida sarcoma
1945	Hiroshima and Nagasaki atomic bomb
1951	Dameshek idea of MPD from HSC
1956	Warburg effect
1958	Quantification of transformation by RSV
1960	Ph discovery
1960	Autotransplantation of human cancer
1963	CML-WBC transfusion to patients with leukocytopenia
1965	G6PD leiomyoma
1967	G6PD CML
1970	Abelson murine leukemia virus
1975	Splenectomy in CML
1978	Stem cell niche idea
1985	BCR-ABL in CML
2001	Imatinib

antiapoptotic activity confer an ability to escape apoptosis on tumor cells. Since long ago, it has been known that tumor cells can survive in hypoxic and low nutrient conditions because of their specialized metabolic changes. To summarize, (1) oncogene, (2) tumor suppressor, (3) stabilization of pathological proteins, (4) cell death escape, (5) endless mitosis, and (6) altered metabolism are the main properties of tumor cells themselves. While those features can be analyzed *in vitro*, tumors are “abnormal organ-like structures” containing a mixture of tumor cells and seemingly normal host cells. The two important host elements are (7) stroma with vessels and connective tissues and (8) immune cells. Genomic instability in tumor cells is not only the cause but also the result of the pathological alteration in (1–8). Tumor-linked inflammation and metastasis are interdependent and cannot be discussed without the information on (1) through (8).

In Part III, I will describe solid tumors and leukemias in clinical settings in Chap. 9, and after describing the aforementioned features of tumor cells (1–6) in Chap. 10 and then tumor microenvironment with information on (7) and (8) in Chap. 11, I shall discuss experimental systems of tumor progression, especially tumor invasion and metastasis in the body in Chap. 12. The inflammatory features of cancer are embedded in Chaps. 11 and 12, and genomic instability is described in each place suitable for description.

9.2 Origin and Development

The most clinically relevant information should be directly obtained from patients. Elucidation of mechanisms is supported by the working hypothesis based on experimental results, which should be repeatedly challenged along with the progress of medical science. The origin of cancer is not an exception.

The origin of cancer has at least three key aspects. The first is the physiological [1] cell type originally hit by cancer-causing cues, the second is the [2] time-dependent development of the hit cells, which are now equivalent to cancer-initiating cells, during the clinical course of the cancer patient, and the third is the [3] spatial position of the hit cells in a body.

9.2.1 *Cell Type*

Each organ or tissue has tissue-specific stem cells and cells that are differentiated from them, including proliferating progenitors and differentiated quiescent cells. The differentiation is destined for physiological functions of the cells required for the organ homeostasis. Stem cells can hardly grow actively maintaining quiescence but self-renew and give rise to progenitors upon appropriate stimuli. This raises a fundamental question of at which level of cell differentiation oncogenic cues hit the cells. Multiple cell types within the same organ or tissue could undergo malignant transformation. Whereas the transformation of osteoblast within a bone is responsible for osteosarcoma, that of hematopoietic cells that also are localized within bones causes leukemia.

9.2.2 *Time*

The hit cells initiate the cancer program described in the overview in a time-dependent manner. Malignant cells within a tumor may not be the same on the next day irrespective of cell division. They may differentiate, acquire migrating or more growth-promoting capability, be damaged to undergo necrosis or apoptosis, which in turn transmit extracellular signaling toward surrounding microenvironment. The reciprocal stimuli between hit cells and microenvironment proceed to add more changes to both of them. Ideally, clinical course of cancer needs to be frequently monitored by convenient and noninvasive methods, including blood test and imaging studies. Serum biomarkers often are used in clinical settings to evaluate the effectiveness of treatment and prognosis.

9.2.3 *Space*

The third aspect has a close linkage to cell migration within a body. If the hit cells have a single fixed spatial position within a body, the resulting cancer appears and stays there. A well-known bone tumor osteosarcoma most commonly affects the long bone with preferential metastatic progression to lung and brain. Without metastasis, it may stay at the end of the right femoral bone and you never observe cancer cells in the left bone. This contrasts well with leukemia. Hematopoiesis also takes place within bones. Although diagnosis of leukemia is routinely made under clinical inspection of bone marrow from sternal or iliac bone, the same results should be found with that from right and left bones of the same patient. Detection of leukemic cells at different spatial positions at the same time indicates either cancer-causing hits at multiple spatial positions in a body or leukemic cells can migrate throughout the body and stay by growing in the same bone microenvironment but different sites.

9.2.4 *Clonality*

To understand those three aspects, we need convenient signatures pertinent to the cells of interest, which are usually genetic alterations and changes in expression levels or their pattern as easily monitored by qPCR, and expression patterns of cell surface marker antigens that also can be analyzed by flow cytometer. The idea about cancer clonality is almost 50 years old. In 1965, analysis of the variants A and B of the X-linked glucose-6-phosphate dehydrogenase, which are distinguishable from each other by electrophoretic mobility, in 86 samples of normal myometrium and 27 leiomyomas from 5 heterozygous women, clearly revealed that normal myometrium samples that consist of a mixture of uterine myocytes expressing either A or B type showed both A and B, but each tumor displayed either A or B [1]. This suggests clonal origin of each of the leiomyomas by negating the presence of both A and B types within the tumor.

Two blood cancers, chronic myeloid leukemia (CML) and multiple myeloma (MM), are good examples of clonal tumors, because the hematopoietic system has a reliable differentiation scheme that can be easily recognized by morphology and molecular signatures. Both CML and MM can provide a nice frame for understanding the aforementioned three aspects. We could apply what we learn from those blood cancers to solid tumors to understand their mode of clonal origin and progression.

9.3 Physiological Hematopoiesis

9.3.1 General Information

In bone, there exist cellular components called bone marrow with a very small amount of interstitial matrix. Therefore, marrow obtained by passage through syringe needle can be mechanically dissociated with ease for single cell suspension. This is useful for diagnosis of blood diseases by bone marrow aspiration in clinical settings. Bone marrow cells are composed of two distinct lineages of primitive cells: blood cells derived from hematopoietic stem cells (HSC) and stromal cells that originate from mesenchymal stem cells (MSC). The most prominent common feature of HSC and MSC is that both have a potential to give rise to a variety of cells that can be clearly distinguished by morphology and biochemical properties. When a bone marrow suspension is plated onto dishes, stromal cells adhere to the bottom of the dishes and discriminated from nonadherent blood cells by repeated washing. Depending on the culture conditions, those cells form distinct colonies. MSC was initially described by Alexander Jakovlevich Friedenstein in 1970 as colony-forming unit-fibroblast (CFU-F) in monolayer culture of guinea-pig bone marrow, which could be induced to form bones [2]. Blood cells include white blood cells, red blood cells, platelet, and their progenitor cells hierarchically organized as shown in Fig. 2.1 in Chap. 2. Osteoclasts are differentiated from HSC- GMP by stimulation with M-CSF, TNF α , and RANKL. The progeny of MSC include fibroblasts, endothelial cells, adipocytes, osteoblast, and chondrocytes, all of which contribute to the maintenance of bone architecture and cooperate with blood cells.

HSC are rare population; for example, LSK and LT-HSC (see below) represents approximately 0.05 % and 0.007 %, respectively, of nucleated bone marrow cells. They can self-renew to generate the same cells. Non-self-renewing multipotent progenitors (MPP) upon exposure to specific stimuli give rise to common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). CMP have two lineages to diverge: granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP).

At least three paths branch from CLP, pro-T, pro-B, and proNK. Pro-B cells differentiate to naïve B cells in bone marrow by V(D)J recombination eventually presenting IgM on the cell surface. This is an ordered rearrangement of the variable (V), diversity (D), and joining (J) gene segments of the immunoglobulin (Ig) genes. Both Ig heavy and light chains have those segments and rearrangement of light chain follows that of heavy chain. The naïve B cells enter the lymph node to be stimulated by DC, which allows clonal expansion after class switch recombination (CSR) and somatic hypermutation (SHM). CSR changes the isotype of Ig, including IgG, IgA, etc. SHM introduces point genetic mutations in the already rearranged Ig genes to generate high-affinity antibodies. The series of molecular events are called germinal center (GC) reaction. The expanded B cells then go back to bone marrow for further growth. B cells are destined to produce Ig after making contacts with DC. Pro-T cells go to thymus to be mature T cells. Thymic regeneration changes the

responsible site for T-cell division source dependently on time after birth due to age-related thymic retrogression in which thymic epithelial cells decline as early as 1 year after birth. In healthy human adults, 90 % of the naïve CD4⁺ T cells are generated by T-cell proliferation in the periphery but not in thymus.

9.3.2 *Methods to Assay Self-Renewing Ability*

HSC is defined by their potency to reconstitute the entire hematopoietic system in a serial manner in lethally irradiated recipient mice. Frequency of HSC (Lin⁻Kit⁺Sca1⁺CD150⁺CD48⁻) in bone marrow from adult mice is reported to be <1:20,000 (0.005 %).

Human markers to isolate by fluorescence activated cell sorting (FACS) and evaluate the isolated fraction of cells to determine the hierarchical position are (Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁻) for HSC, CD34⁺CD38⁺IL⁻3Rα⁺CD45RA⁻ for CMP, (CD34⁺CD38⁺IL⁻3Rα⁺CD45RA⁺) for GMP, and so forth. The assay to confirm the differentiation potentials to myeloid lineage is to culture the cells in the presence of appropriate cytokines. The ability to self-renew and reproduce the determined progeny is assessed in vitro by culturing the cells in hydrocortisone-supplemented media on irradiated M2-10B4 monolayers for 6 weeks before plating into methylcellulose media for 2–3 weeks further incubation. The final readouts are morphology-based colony counts. A well is scored as positive when colony-forming unit-granulocyte/macrophage (CFU-GM), CFU-granulocyte/erythroid/macrophage/megakaryocyte (GEMM), or burst-forming unit-erythroid (BFU-E) is observed. This is called long-term culture-initiating cell (LTC-IC) assay, which was reported by Heather J. Sutherland in 1989 [3]. Myeloid progenitors have almost no self-renewing ability as shown by the group of Irving L. Weissman that the estimated frequency of LTC-IC was 1/20 for Lin⁻CD34⁺CD38⁻ cells, 1/1700 for CMP, 1/4600 for GMP, and 1/5800 in MEP [4].

Alternatively, the isolated cells are transplanted to sublethally irradiated NOD/SCID mice [5]. Irradiation eliminates the capacity of all bone marrow cells to contribute hematopoiesis. If the transplanted cells can home back to or repopulate bone marrow, survive in the recipient microenvironment and reproduce their progeny, the transplanted mice can survive with repopulated progenitors and functionally differentiated cells. The reader should notice that this in vivo assay may involve human recipient cells into murine host. Such marrow-repopulating cells were termed SCID-repopulating cells (SRCs). Instead of NOD/SCID that still retain endogenous NK cell activity high enough to eliminate injected cells, especially human short-term repopulating hematopoietic cells that reconstitute both myeloid and lymphoid for <4 months, NOS/SCID-β2 microglobulin-null mice and NOD/SCID/IL2Rγ-null (NSG) mice also are utilized for the potential stem cell candidate to have enhanced self-renewing ability [6].

Experimental Immunodeficiency Mice

Clinical samples are not only subjected to measuring instruments, such as flow cytometer, but also animal assays, as described above, although they are not commonly used as a routine laboratory test. To overcome the immunorejection due to interspecies differences the animals are usually deficient in immune system. I briefly explain their characteristics, because they are, needless to say, also useful in basic research.

Nude mice are of spontaneously occurrence on the background of C57BL/6 strain, hairless, and defective in thymus development due to homozygous deletion of transcription factor FOXP1. Lack of thymus abrogates the generation of T-cell population. C57BL/6 with black hair and BALB/c with white hair are two main sources of laboratory mice. Severe combined immunodeficiency mice (SCID) were discovered during the quantitation of serum immunoglobulin levels [7].

SCID mice display autosomal recessive inheritance of defect in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) with several backgrounds, including C57BL/6. SCID mice lack both T- and B-cell functions but retain DC, myeloid cell, and NK functions. There is leakiness and difference in those functions in a species-dependent manner.

The beige mice have a mutation in the LYST gene that is responsible for proper functions of perforin in both NK cells and CTL [8]. Thus, the mice are recognized as defective in NK cells. SCID/beige mice are defective in T, B, and NK cells and utilized in tumor implantation experiments.

NOD means nonobese diabetic mice of spontaneous development and 18 genetic hot spots for defect are known, including complement C5 and class 2 MHC [9]. NOD mice are not only diabetic, because CD8 T cells target pancreatic β cells, but also impaired in the functions of NK cell, macrophages, and complement. NOD/SCID mice have both phenotypes of NOD and SCID, thereby lacking both acquired and innate immunity [5]. NOD/SCID/B2m-KO mice have further defect in β 2-microglobulin (B2m) that results in impaired CD8 and NK cells since B2m participates in the formation of class I MHC to recognize between self and non-self (see Chap. 5) [10]. NOD/SCID/IL-2Rcommn γ -KO (NSG or NOG with NOD sources from Jackson or Shionogi, respectively) mice have defect in the γ chain that is common to IL2R, IL4R, and IL7R, which impairs both T and B cell functions [11, 12].

9.4 CML

9.4.1 General Information

CML has been recognized as a stem cell disease, which means that this cancer originates from or its direct cause takes place in hematopoietic stem cells. Based on the careful clinical observation, William Dameshek proposed in 1951 a conceptual revision of CML, polycythemia vera, and erythroleukemia, in which the types of growing cells are myeloid cells, mature erythrocytes, and immature erythroblasts, respectively, from proliferative diseases of just one cell type with occasional variants to myeloproliferative diseases with pancytosis due to hypothetical myelostimulatory factors without drawing any clearcut lines among them [13]. This I believe is indeed the beginning of the idea of stem cell origin of the blood diseases.

The most prominent features of clinical manifestation in CML is massive expansion of myeloid progenitor cells with every stage of differentiation in chronic phase (CP), which is inevitably followed by intractable acute leukemic progression phase called blast crisis (BC). The duration between CP and BC was 3–5 years after diagnosis before the advent of tyrosine kinase inhibitors (TKI) of current clinical use. More than 80 % of CML patients are estimated to survive after 8 years under TKI treatment. I explain CML from the view of the three aspects of the hit cells: (1) cell type, (2) time, and (3) space. Those three aspects are interconnected with each other.

9.4.2 Cell Type

9.4.2.1 HSC: GMP: preB

To address to those issues, CML has its own golden marker ideal for molecular signature, the Philadelphia chromosome (Ph) that is generated by reciprocal translocation between chromosome 9 and 22, t(9;22)(q34;q11). This juxtaposes the ABL tyrosine kinase gene on chromosome 9 and the BCR gene on 22, which results in the production of the constitutively active tyrosine kinase fusion oncoprotein BCR-ABL. The discovery of Ph is back in 1960 by Peter Nowell [14]. He stated in his article in 2007 recollecting the discovery that “I knew nothing about cytogenetics at this time but felt that the chromosomal preparations of the leukemic cells warranted investigation for any abnormalities. I found no one on our campus interested in human chromosomes.” Readers can imagine how impressive the initial finding was at that time.

Nowadays, CML patients are treated with success by BCR-ABL tyrosine kinase inhibitors (TKI), such as imatinib. Their efficacy to achieve complete hematological response (platelet count $<450 \times 10^9/\text{L}$, white blood cell count $<10 \times 10^9/\text{L}$, no

immature granulocyte and basophile $<5\%$) is close to 100%. To monitor the disease progression, RT-qPCR analysis to measure the ratio of BCR-ABL relative to ABL as a control gene has been utilized as the most sensitive clinical test. Major molecular response (MMR) is BCR-ABL/ABL $<0.1\%$ with the limit of detection of 0.001% as undetectable molecular residual disease (UMRD) or complete molecular response. Complete cytogenetic response (CCyR) of approximately 1% also is useful. CML is currently treated with those levels of residual disease.

9.4.2.2 Clonality

Even before the discovery of BCR-ABL, Ph served as a good signature for CML to prove its clonality. The G6PD heterogeneity of electrophoretic mobility subtypes A and B was applied as described above to CML to confirm its clonal origin [15]. It revealed that single phenotypes were detected in erythrocytes, platelets, and cultured macrophages but not fibroblasts. Another germline heterogeneity to demonstrate the clonality of CML also was applied to CML. The mosaic Turner syndrome manifests a mixture of 45X and 46XX karyotype [16]. Mosaic Turner syndrome with CML with the original 45X+Ph karyotype changed to normal 46XX without Ph after the effective chemotherapy for lymphoblastic crisis, demonstrating the persistent existence of normal sex chromosome clones of HSC that await reactivation when normal hematopoiesis could be restored.

We should be aware of the logics of germline versus somatic (acquired) genetic affairs. G6PD heterogeneity and Turner mosaic exist at conception and Ph is acquired after birth. While G6PD heterogeneity is transmitted to offspring, somatic mutation, such as Ph is not passed onto children. Therefore, if the acquisition of Ph takes place in multiple cells independently at the same time or at least during a certain period of time of hit, Ph-bearing, namely leukemic, cells should have a mixture of the type A and B in G6PD, or 45X and 46XX in mosaic Turner. The detection of Ph only in the background of either of those for each heterogeneity shows that Ph-positive leukemic cells are of clonal origin.

9.4.2.3 Stem Cell Origin

The evidence to show the involvement of erythroid lineage came from the experiment in which CML bone marrow cells were incubated with radioisotope ^{59}Fe , which labels only erythroid cells and coexistence of radioactivity and Ph was analyzed (Fig. 9.1). Furthermore, detection of Ph in all lineages of blood cells, including erythroblastic, myeloblastic, megakaryoblastic, and lymphoblastic progeny, as well as in the HSC clearly demonstrated its origin in HSC [17]. Ph was detected on osteoclasts derived from HSC but not other types of cells, such as fibroblasts that constitute bone marrow stroma [18].

Back to the definition of HSC, we need evidence by which to show CML-HSC can reconstitute the whole disease. An invaluable report in human was made in

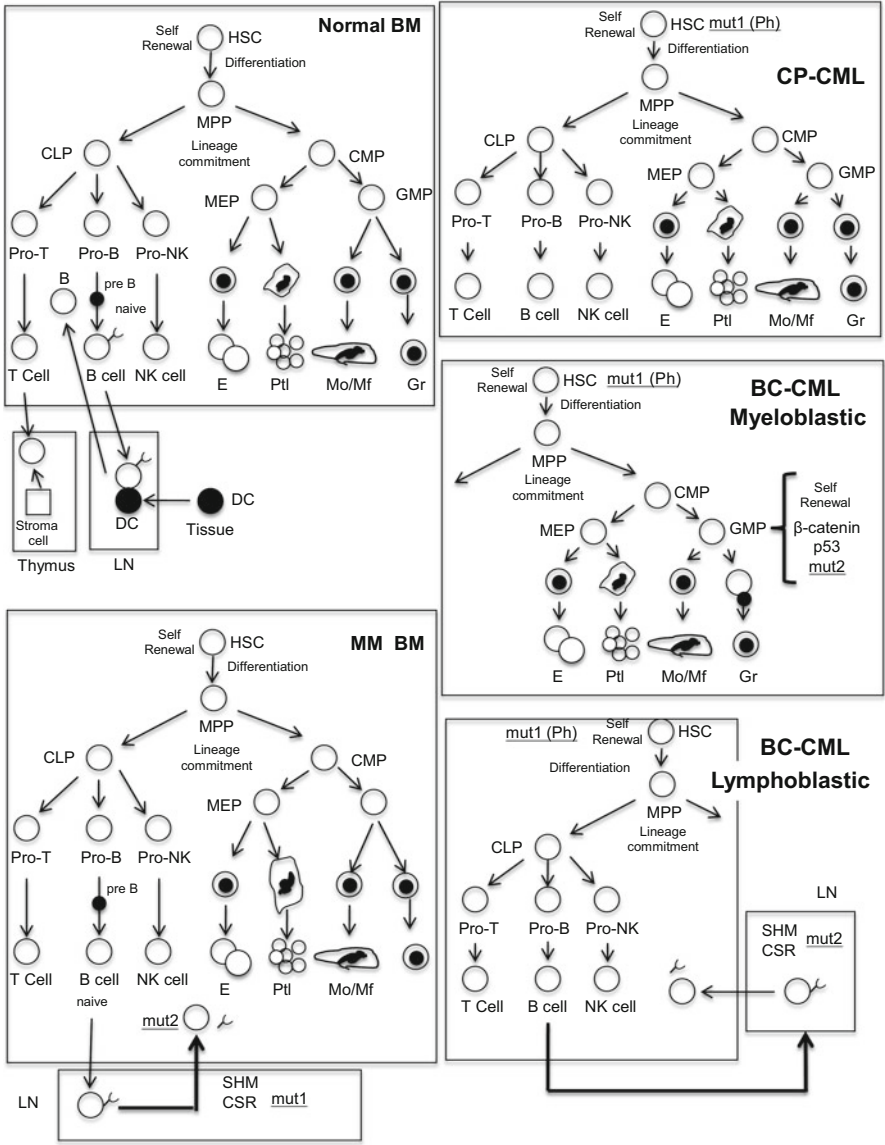


Fig. 9.1 CML and MM. Scheme shows where in the differentiation process leukemic activities take place. Ph is the first mutation (mut1), which is followed by the second mutation (mut2), such as that in p53. See test for SHM and CSR that promotes acquisition of mutations in lymph nodes (LN)

1963 on the persistent growth of CML cells after white blood cell transfusion to a postchemotherapeutic AML patient suffering from leukopenia [19]. In those days white blood cells in CML, whose numbers in the peripheral blood is sometimes >

100,000 per cubic millimeter, were utilized for transfusion for neutropenic patients, because it is even now difficult to obtain a large number of neutrophils for transfusion. Ph-positive cells were detected in 99 % of bone marrow, including erythroid cells 29 days after transfusion in one patient and Ph-positive cells were found in 5 % bone marrow cells 54 days after transfusion in another, strongly suggesting that CML-HSC is circulating in the peripheral blood and can make an engraftment.

The activity of CML-HSC from chronic phase is exhausted in both LTC-IC assay in vitro and NOD/SCID in vivo [20]. To facilitate their ability, transduction of NA10HD homeodomain fusion gene in those population of cells or application of NRG mice are of current strategy.

Thus, HSC is hit in CML allowing clonal expansion but by differentiating. It is noteworthy that normal differentiation program is not abolished in CML. However, BCR-ABL-expressing clonogenic and LTC-IC-derived progenitors can be detected in patients under sustained undetectable molecular residual disease.

What hits HSC to cause CML?

One precious and reliable fact in human should be what was described in the review of 45 years study of survivors of Hiroshima and Nagasaki atomic bomb in 1945 (Table 9.1) [21]. The CML occurrence dominates over other leukemia and peaked around 6 years after exposure irrespective of the dose of irradiation with an estimated apparent threshold 0.5–0.09 Gy. The average chest X-ray gives an absorbed dose of 0.0001 Gy in the lungs. The atomic bomb also increased breast cancer incidence. Currently, it is an alleged notion that X-ray causes genetic mutations. We should remember the work by H.J. Muller, which first showed mutations could occur by external stimuli in *Drosophila melanogaster* back in 1927 [22].

9.4.3 Time Onset: CP: BC

The easiest way to see if a patient is in remission of CML, for example, by imatinib is to confirm that white blood cell numbers in peripheral blood are within normal range. Leukemic disorders can be monitored by routine blood tests.

When does the leukemic transformation by BCR-ABL take place to cause CML? Given that CML patients can stay apparently healthy in the chronic phase, it is difficult to estimate the leukemic onset of CML. By using 2-step RT-PCR in which 10^8 cells per sample were tested in 40 replicate reactions, detection of BCR-ABL fusion gene products were documented in peripheral leukocyte samples in 27 % of healthy subjects and non-CML leukemic cell lines, such as HL60 and KG1 [23, 24] (Fig. 9.2). Although CD34⁺ cord blood cells with retrovirus-mediated forced overexpression of BCR-ABL were sufficient to cause CML-like manifestation in NOS/SCID mice, CML-HSC from chronic phase patients have poor reconstituting ability in both LTC-IC assay and standard transplantation into NOD/SCID mice. Moreover, we observed that lentivirus-mediated transduction of CD34⁺ marmoset

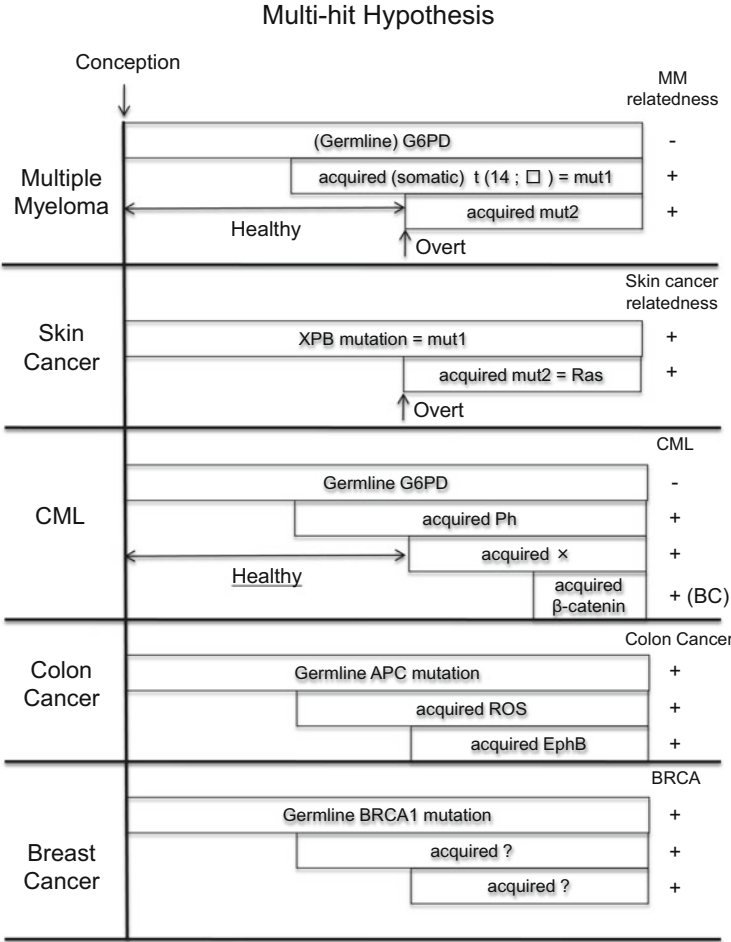


Fig. 9.2 Multi-hit hypothesis in carcinogenesis. Affected individuals are seemingly healthy until they acquire mutation(s) crucial for the cancer progression. The germ-line mutation is known for colon cancer with APC mutations. Morphologically, precancerous adenomas develop after an additional K-Ras mutation, which is sequentially followed by further acquisition of mutations in p53 or SMAD4 to generate adenocarcinomas. This adenoma to adenocarcinoma sequence is followed by the metastatic progression phase, which requires a variety of other accumulation of mutations, including MACC1 in regulation of HGF signaling, EphB, and CD133. Considering first the cancer stem cell, CD133⁺ cells contain the cells that have the ability to phenocopy the original colon cancer in immune-compromised mice in a serially transplantable manner. However, the precise functions of CD133 are not known, and CD133 certainly does not serve as a reliable stem cell marker in the normal colon epithelial cells

cells with BCR-ABL failed to induce leukemia even if BCR-ABL expression was maintained for 18 months. Collectively, it is likely that BCR-ABL alone may not be sufficient to cause robust and progressive leukemia in humans but forced high levels of expression of BCR-ABL in mice is (see Chap. 10).

The chronic phase of CML after the insidious onset is followed by blast crisis (BC), an acute leukemic progression phase just like acute leukemia. Generally, leukemic cells in blast crisis have additional cytogenetic aberrations in more than 80 % of patients, which indicates a high level of genomic instability. The evidence to show that BCR-ABL by itself is a mutagen is described in detail in the next section. This raises again a fundamental question of what type of cells are BC-initiating cells in CML-BC. Isolation of HSC and GMP from CML patients and subsequent self-renewal assays as described above in the method revealed that not only HSC ($CD34^+CD38^-$) but also GMP ($CD34^+CD38^+$) from CML-myeloid BC can self-renew with activation of β -catenin, whereas normal GMP cannot [25]. Requirement of β -catenin in HSC maintenance has been shown in a murine model. In other document, eight of nine patients with lymphoid BC had rearrangements in Ig heavy and light chains. In contrast, cells from myeloid BC showed germ line configuration. This at least clearly shows that additional hits to BCR-ABL, which is supposed to be the first hit, take place at the stage of B-cell precursor.

Acute leukemia-initiating stem-like population of cells is GMP in CML-myeloid BC. This contrasts well with the situation in acute myeloid leukemia (AML). In transplantation to nonobese diabetic mice with severe combined immunodeficiency disease (NOS/SCID mice), $CD34^+CD38^-$ cell populations purified in vitro from a variety of morphologically distinguishable subtypes AML patients with FAB subtype M1, M2, M4, and M5 could differentiate in vivo to manifest the same leukemic phenotypes as observed in the patients, suggesting that this population of cells are leukemia-initiating cells, target cells for leukemic transformation [26]. Failed engraftment was reported in the $CD34^+CD38^-$ population of cells derived from M3, acute promyelocytic leukemia. Presence of the leukemogenic PML-RARA fusion gene in the $CD34^+CD38^+$ but not $CD34^+CD38^-$ population of cells indicates that the hit cells in M3 is not primitive HSC [27].

To summarize, transformed cells still undergo further alterations, genetic ones in particular, to become more malignant for the affected individuals. This is an extension of multistep carcinogenesis, meaning that the two malignant phenotypes, growth and cell movement within the body, are aggravated during the clinical course of cancer. Why not improved? Readers shall understand why in Chap. 10.

9.4.4 *Space: Bone Marrow and Spleen*

What governs the extent of cancer distribution in the body depends on its repopulating ability. Even if cancer cells spread to other organs or tissues, but they undergo apoptosis or permanent cell-cycle arrest, no life-threatening affairs can happen. For CML-initiating cells to repopulate in bones of other parts of the body or spleen, CML-HSC needs to make an entry into circulation as blood cells usually do in physiological conditions and resettle and restart growth and differentiation there. This is the reason that you can make a diagnosis of CML whether you

aspirate bone marrow from sternal or iliac bone. We can assume bone marrow that is located within bones in many parts of the body as a single organ with almost the same anatomy and function while we have a single liver for which no other organ or tissue can compensate due to the perfect difference in the components of the microenvironment.

What about the spleen? Splenomegaly is one of the most prominent features of CML and the enlarged spleen is where the extramedullary (outside of bone marrow) hematopoiesis takes place. Postnatal function of spleen in humans is to serve as a dispensable lymphoid organ that filters old or abnormal red blood cells in red pulp and have immunological activities, such as processing of antigens in white pulp. The spleen of CML is diffusely enlarged with a gross granulocytic infiltrates in the presence or absence of codominant growth of megakaryocytic and/or erythroid lineage of cells. A study of splenectomy in nonselected 26 CML patients in CP remission with old antileukemic drug busulfan showed decreased incidence of BC compared with the expected one in patients under satisfactory response to busulfan with two patient surviving after 8 years [28]. I do not think that this was an anecdote in 1975. Given that spleen size is a statistically significant risk factor to achieve CCyR at 18 months, I believe that the work adds precious evidence in human that splenic populations of CML-HSC and CML-GMP presumably with further genetic alterations were subjected to physical elimination by surgery to eventually postpone BC. An experimental evidence with SCLtA/BCR-ABL mouse model showed that splenic CML cells are more resistant to tyrosine kinase inhibitors than bone marrow CML cells (see Chap. 10 for details) [29].

In another well-known blood disease with splenomegaly is myelofibrosis (Mf) in which hematopoiesis occurs to compensate for the defective hematopoiesis due to bone marrow fibrosis [30]. Transplantation experiments of CD34⁺ cells from spleen of Mf patients displayed generation of both myeloid and lymphoid cells that could serially engraft the next recipient mice. This indicates that HSC-Mf or normal HSC exist in the spleen.

It is likely that CML-HSC repopulate in the spleen to expand their progeny as in the case of bone marrow. Then, why does it seldom happen in physiological circumstances? MSC present in bone marrow also may participate in the formation of stromal microenvironment in spleen preparing soil (CML-HSC niche) for seed (CML-HSC) by making an entry into circulation to reach spleen.

9.5 Multiple Myeloma

9.5.1 Cell Type

B cells are affected first in lymph nodes where somatic hypermutation (SHM) and class switch recombination (CSR) are actively taking place. Because Ig heavy chain locus is located on human chromosome 14q32, aberrant translocations in multiple

myeloma (MM) commonly include t (11;14), t(14;14), t(16;14), and t(6;14) [31]. Secondary hit(s) happens in bone marrow after clonal expansion in lymph nodes (Fig. 9.2). The t (14; 18) generating Bcl-2/IgH rearrangement was reported to be detected in 39 of 204 (24 %) healthy individual blood donors in which the breakpoint distribution was comparable to those found in follicular lymphoma, supporting the multistep myeloma transformation process [32]. The absence of clone to clone variation in the Ig genes clearly shows the clonal origin of MM. Thus in MM, the cell type of hit cells is rather mature B after GC reaction but still proliferating in bone marrow without disturbance of maturation as Ig-producing B cells.

It is noteworthy to state that thalidomide when taken during pregnancy had a tragic outcome, i.e., phocomelia due to its teratogenic effects. Thalidomide reemerged as an antiangiogenesis agent as initially proposed by Judah Folkman [33]. Currently, including its more potent analogs, such as lenalidomide and pomalidomide, thalidomide was shown to be useful for the treatment of multiple myeloma by binding to cereblon and thereby enhancing ubiquitination and subsequent degradation of Ikaros (IKZF1) and Aiolos (IKZF3)—two zinc finger transcription factors essential for B-cell development [34].

9.5.2 *Space and Time*

The MM stem cells should be able to repopulate in bone marrow of other bones. They rarely display solid B-cell tumors, such as solitary plasmacytoma of bone or plasma cell leukemia secondary to MM with more than 20 % circulating plasma cells in the peripheral blood. The defect in an adhesion molecule CD56 may result in the loss of anchor to the bone marrow stroma. As in the case of CML-BC, more than 80 % of the plasma cell leukemia patients have aneuploidy, an abnormal number of chromosomes, again indicating high genomic instability in the cells that undergo leukemic transformation [35]. While CML cells are found in bone marrow, spleen, and circulation, MM cells are in bone marrow. Neither of them can invade into other organs, such as lungs. Different from osteosarcoma, MM cells can be aspirated from bone marrow. Therefore, genetic instability may allow extramedullary export but without target organs to resettle and repopulate. One of the convenient clinical biomarkers of MM is serum levels of Ig that can be easily measured by routine laboratory test.

9.6 Tumor

Let's summarize what we have learned from CML and MM, especially on spatio-temporal aspects of hit cell types for cancer development.

1. Hit cells grow and may self-renew and differentiate
2. Multistep hits make the vague outline of the picture of carcinogenesis
3. Cancer cells may repopulate at distant sites via bloodstream

Based on the information, let us try to understand tumors of epithelial origin, including colon cancer, breast cancer, hepatic cancer, lung cancer, prostatic cancer, pancreatic cancer, etc. A critical difference between CML and those cancers is that discrimination of cell type hit for carcinogenesis in those cancers can hardly be made. However, one clear common feature is the temporal and spatial heterogeneity of transformed cells as judged by genetic alterations at least. An old study from Sloan Kettering Institute undertaken in 1960 to show engrafting potentials of minced pieces of primary tumor biopsy samples in subcutaneous tissues of the same patients happened to reveal that it was possible but with low efficiencies, although the study was small and uncontrolled and I believe it certainly would cause ethical problems these days [36]. This autotransplantation experiments in humans is a forced metastasis to skin and clearly indicates that spatiotemporal progression of cancer could be supported by cancer-initiating cells within the transplanted specimen as in the case of transfusion of white blood cells from CML patients.

Unfortunately, the degree of heterogeneity in those cancers is by far larger than that in CML-CP. Therefore, the successful results have been obtained by analyzing a rather selected or homogeneous population of patients in cancer-prone families with genetic alterations suffering from a certain type of cancer.

9.7 Terms in Genetic Epidemiology

Before plunging into the solid tumor issue with so much genetic information, we need to understand epidemiological terms correctly. An article entitled, <My Medical Choice> in The New York Times by actress Angelina Jolie described her own genetic problems with encouragement for other women [37]:

...My mother fought cancer for almost a decade and died at 56...the truth is I carry a “faulty” gene, BRCA1. ...Only a fraction of breast cancers result from an inherited gene mutation. Those with a defect in BRCA1 have a 65% risk of getting it... On April 27 (2013), I finished the three months of medical procedures that the mastectomies involved. ...On a personal note, I do not feel any less of a woman... The cost of testing for BRCA1...at more than \$3,000 in the US...remains an obstacle for many women...

The article by Angelina Jolie attracts many people, because it appears to be of artistic fulfillment. The article contains medical facts, fear, love, encouragement, even economy.

She suffers from an inherited breast and ovarian cancer syndrome but is still healthy as a mutation carrier. Similar to the heterogeneity of G6PD in the CML section, the BRCA1 mutation here is on the germline, meaning that the mutation is found in all the cells in the body, and inherited from parent to their offspring

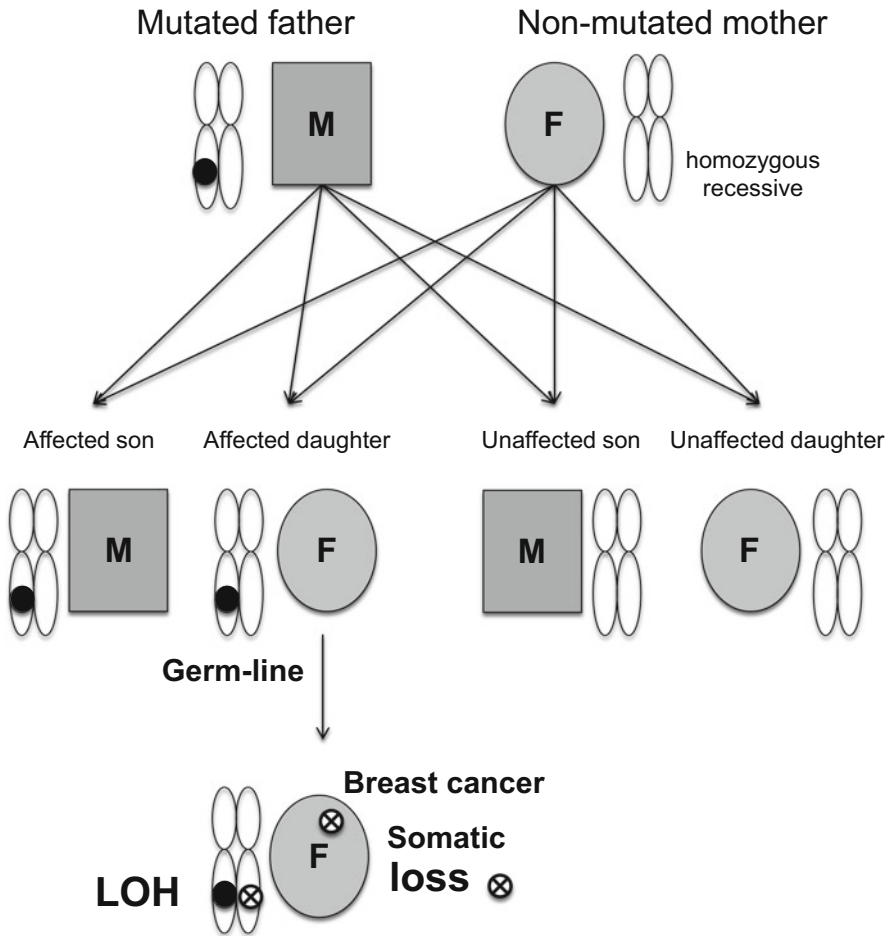


Fig. 9.3 Hereditary germ-line mutation and LOH. One mutated allele causes cancer risk that is inherited in an AD fashion from either parent. The mutated father (M male) is heterozygous for the mutation. Loss of heterozygosity (LOH) is loss of functional copy of tumor suppressor causing high-risk mutation

[38, 39]. We have two alleles for each gene and typically either one of the two alleles is dominant to function for the production of the gene product (Fig. 9.3). Mutation does not necessarily result in defect of the gene product but instead may activate it (see the p53 case in Chap. 10). BRCA1 has mutations of the former type and is defined as tumor suppressor gene as explained by the following.

One BRCA1 mutated allele on human chromosome 17 causes breast cancer risk or predisposition that is inherited in an autosomal dominant fashion from either parent [40]. Although the mutation reduces or inactivates the BRCA1 gene functions, the predisposing allele behaves as a recessive allele in somatic cells. In breast cancer family, cancer cells invariably lost the wild-type allele, the BRCA1

nonmutated allele, which took place in the lifetime as a somatic mutation. Therefore, we need to understand the sequential events in the genome, a germline inactivating mutation on one allele first causing predisposition, secondly somatic inactivating mutation on the other allele, and this makes one strong step toward breast cancer. It should be noted that mutations of other genes, such as p53 (see below), also took place in breast cancers arising from BRCA1 germline mutation carriers.

Generally, one allele from father and the other allele from mother occasionally have some differences in DNA sequence as represented by microsatellites. This heterozygosity is sometimes lost by genetic deletion of the locus in cancer, which is called loss of heterozygosity (LOH) (Fig. 9.3). The above-mentioned second mutation of BRCA1 superimposed on the first germline mutation is an example of LOH. Even in sporadic cases of breast cancer, LOH has been reported not only in BRCA1 (~30 %) but also in ATM (50–60 %) [41]. The frequency of LOH seems to depend on the type of cancer. The ATM LOH is observed in, for example, Hodgkin lymphoma, which is usually sporadic, but infrequent. ATM is a responsible gene for ataxia-telangiectasia, whose clinical traits are inherited in an autosomal recessive manner. Homozygotes are prone to develop a variety of cancer. However, epidemiological analysis has revealed fivefold breast cancer predisposition in ATM heterozygotes.

In dominantly inherited cancer-prone Li-Fraumeni syndrome with germline p53 mutations, somatic mutation of LOH in the p53 locus occurs similarly to that in BRCA1 [42, 43]. Osteosarcoma cells of the affected individual had both of the germline mutation of one allele and loss of the other allele, whereas adjacent normal tissue and skin fibroblasts had only the germline mutation with the opposite allele being normal.

Although both breast and ovary cancers exclusively affect women, affected subjects can be male. However, if mutations happen to be set in both alleles by inheritance from affected mother and affected father, it is embryonic lethal.

Only a fraction of breast cancers result from an inherited gene mutation. This is the frequency of BRCA1 mutation in breast cancer population in general and is called prevalence, ranging from 3.3 % in American women to 11.7 % in the Ashkenazi Jewish population. A portion of people with breast cancer in a mutated BRCA1 population is called penetrance. A review of 223 unrelated families with BRCA1 mutations revealed that breast cancer penetrance to 70 years of age was 68 %. BRCA1 allele mutation is highly penetrant. Incidence is another epidemiological term to express new cases that arise in a certain population in a given period of time.

The presence of BRCA1 mutation does not always result in breast and ovary cancer. A variety of types of BRCA1 mutation have been detected so far with high-versus low-risk mutations. As described in detail in the next section, BRCA1, ATM, and p53 all participate in DNA repair. BCR-ABL in CML also impairs DNA repair. Breast cancer with BRCA1 has the first germline hit at conception, whereas CML has Ph as the first somatic hit later in life. However, both cancers become overt as a consequence of further hits. Although application of statistical

analyses to 48 cases of retinoblastoma made Alfred G. Knudson hypothesize in 1971 that the disease is caused by two mutational events, the idea of incessantly occurring mutations in cancer by the normal molecular collisions was originally given by Timofeeff-Ressofsky in 1935 [44]. Given that DNA homeostasis is governed by constant sensing and fixing of damaged DNAs in physiological as well as pathological settings, it safely can be said that abrogation of DNA repair in both activation of BCR-ABL and inactivation of BRCA1 reasonably leads to genomic instability that always accompanies cancer progression.

9.8 Breast Cancer

9.8.1 *Cell Type*

As stated earlier, prevalence of BRCA1 mutation is roughly 5–10 % in the whole breast cancer population, indicating that majority of breast cancer is caused by other genetic alterations. The human mammary gland contains stromal cells, such as endothelial cells and epithelial cells of two phenotypes that are conveniently distinguished by immunohistochemical analysis, keratin 8/18-positive luminal type and keratin 5/14-positive basal type [45]. Although the BRCA1 mutation is found in all cell types, physiological expression of important genes in breast homeostasis, such as estrogen receptor (ER), is cell-type-dependent. Depending on before or after menopause, an endocrine therapy targeting estrogen-ER is planned.

Approximately 7 % of the entire epithelial cells are ER-positive and 87 % of ER-positive cells are luminal epithelial cells. Puberty provokes proliferation of mammary epithelial cells in response to estrogen [46]. ER-positivity is detected in 75–85 % of breast cancers [47].

The discovery of viral oncogenes that originated from mammalian counterparts prompted many researchers to analyze human cancer samples. HER2 was not an exception. HER2 was isolated as a homolog of epidermal growth factor receptor EGFR tyrosine kinase, whose viral counterpart oncogene is v-erbB in which the tyrosine kinase activity is constitutively activated and was alternatively called EGFR2 or neu oncogene. With 189 primary breast cancer samples, Dennis J. Slamon showed in 1987 that amplification of HER2 with 2- to more than 20-fold copies of DNAs was found in 30 % and this correlated well with more than three axillary lymph node metastases but never with ER positivity [48]. As discussed in detail in the next section, HER2 in breast cancer is only partially translated. In other words, in contrast to BCR-ABL in CML described above, the mechanisms of carcinogenesis by HER2 are not reasonably uncovered, although anti-HER2 treatment is accepted as a successful strategy. I believe that even untranslated research is valuable if it results in effective strategy in clinical settings. Since the report by Charles M. Perou in 2000 on the categorization of breast cancer

based on the gene expression profile and subsequent dendrogram, the apparently heterogeneous breast cancer has been grouped into roughly four entities, including (1) normal-like (phenotype with expression profile similar to that in noncancerous normal mammary tissue), (2) luminal-type (with ER-positive), (3) ER-negative (with HER2-positive), and (4) basal-like (phenotype with expression profile of normal myoepithelium, such as CK5/6, CK14, and CK17) [49, 50]. Convenient laboratory examinations to detect ER, progesterone R (PR), and HER2 have given a simpler grouping based on intrinsic immune-reactivity: ER^+HER2^- (luminal A), ER^+HER2^+ (luminal B), ER^-HER2^+ and ER^-HER2^- . What is described as ER^+ is ER^+ and/or PR^+ (positive for either) and ER^- means ER^- and PR^- (negative for both). Therefore, ER^-HER2^- is triple negative $ER^-PR^-HER2^-$. Comparative analysis of 6370 women with triple-negative breast cancer with 44,704 women with other phenotypes showed aggressive nature of the tumor cells and poor survival in triple negative phenotype [51]. To use the clinical term triple-negative, 66–90 % of triple-negative breast cancer, which is 10–24 % of total breast cancer population, is basal-like phenotype that constitutes 16–18 % of all invasive breast cancer. Conversely, most of the basal-type is triple-negative.

The critical point is that transformed mammary epithelia cells grow dependently on both receptors ER and HER2. In the case of ER, estrogen is the growth factor. However, HER2 lacks its own ligand but is still active to transmit growth signaling (see Chap. 10). The current breast cancer therapy is based on this categorization and antiestrogen therapy by tamoxifen, anti-HER2 therapy by trastuzumab or lapatinib, and combination of both, are clinically selected. The triple-negative breast cancer patients are usually treated by conventional chemotherapy.

Of note, patients with BRCA1 mutations tend to be triple-negative. Eunjung Lee reported that 48 % of 46 BRCA1-mutated breast cancer patients were triple-negative, whereas 12 % of nonmutated were triple-negative [52]. Another report by Nadine Tung showed that 68 % of 182 BRCA1-associated breast cancer patients were triple-negative [53].

Breast cancer without invasion to distant organs is ideally resected by surgery. Postoperative chemotherapy is called adjuvant and preoperative is neoadjuvant. A recent report with 1948 patients showed that addition of anti-VEGF (see Chap. 11 for details) reagents that is supposed to inhibit tumor angiogenesis to chemotherapy led to more complete pathological response than the chemotherapy alone (14.9 % vs 18.4 %, $P = 0.04$) and this effect was more prominent in 663 triple-negative patients (27.9 % vs 39.3 %, $P = 0.003$) [54]. However, the role of anti-VEGF therapy in breast cancer is still unclear.

9.8.2 Time

It is known from the past that breast cancer recurrence can take place even after 17–25 years not only in the site of previous surgery but also in distant sites [55]. Then, is it predictable or not until massive recurrence is found in distant

lymph node metastasis? The biomarker could give information of the tumor burden during the clinical course of treatment. In breast cancer, serum levels of CA15-3 or CEA were elevated in roughly 10 % of 740 preoperative patients in stage I–III (see below) [56]. If elevated, the patients can be monitored for the efficacy of treatment and clinical outcome by measuring their levels by easy blood tests.

Generally, biomarkers, such as CEA, reflect disease progression. BCR-ABL causes CML but also its disappearance reflects remission. The well-known and reliable biomarker of hepatocellular carcinoma α -fetoprotein (AFP) is transcriptionally suppressed in adults by ZBTB20, liver-specific knockout of which releases its expression but failed to induce overgrowth of hepatocytes [57]. Readers can see the difference between BCR-ABL and AFP. In addition to those serum biomarkers, expression levels in tumor cells may predict patient prognosis. For example, subtractive hybridization of early-stage non-small lung cancers with versus without subsequent metastasis revealed a noncoding RNA called MALAT-1 (metastasis associated in lung adenocarcinoma transcript) of predictive value. Patients with metastasis had ninefold higher expression levels than those without [58].

We have seen a sequence of genetic alterations in CML, from a single abnormality BCR-ABL in chronic phase followed by additional cytogenetic changes including those of p53 as well as activation of β -catenin. In colon cancers, genetic alterations of p53 are found in the late tumor progression stage in the adenoma to carcinoma sequence. In breast cancer as found in Li-Fraumeni syndrome, it is present from conception as a germline defect. Therefore, although genetic alterations accumulate during the course of tumor progression, their order can vary depending on the tumor type.

Noncoding RNAs

Only 1.5 % of the human genome encodes proteins [59]. The regions between genes are not silent or untranscribed junk DNAs, and more than 85 % of the genome is actively transcribed with varying copy numbers [60]. Those noncoding (nc) RNAs are grouped into small (<200 nt) and long (>200 nt) ncRNAs. Many of them regulate transcription in an epigenetic manner and are involved in many diseases, including cancer and cardiovascular disorder [61]. Small ncRNAs include microRNAs (miRNAs) of ~22 nt and PIWI-interacting (pi) RNAs of 24–30 nt. The above-mentioned MALAT-1 belongs to the long nc (lnc) RNAs. Some miRs are described in differentiation of blood cells (see Chap. 2), homeostasis of hematopoietic stem cells (Chap. 14), and in cancer treatment (Chap. 16).

9.8.3 *Space*

In addition to cell types, readers can now understand that we should consider spatiotemporal aspects. Progression of cancer of epithelial origin is clinically grouped by the TNM mode to determine the clinical stages of the disease. Tumor size (T), lymph node metastasis (N) and distant (hematogenous) metastasis (M) give key prognostic information for clinicians to make a decision on the first step therapy when patients see the doctor, because, needless to say, the mode changes during the clinical course. Of note, systemic metastasis can take place in a manner independent of lymph node metastasis. Already in 1989, it was described by statistical analyses of 24,740 patients that 54.4 % of breast cancer were negative in the regional LN spread at the surgery, but 5-year survival rate was 45.5 % with tumors sized >5 cm and LN-positive, whereas that was 96.3 % with those <2 cm without LN spread [62].

9.8.3.1 CTC-Mediated Hematogenous Spread

To achieve hematogenous metastasis, breast cancer cells need to make an entry into circulation. Presence of certain levels of circulating tumor cells (CTC) is a useful biomarker not only for prognosis but also to make a decision for therapeutic strategy. Epithelial cell adhesion molecule (EpCAM) and cytokeratin 19 (CK19) are frequently used surface antigens that are pertinent to cells of epithelial origin usually absent in circulation to detect CTC by immune-magnetic separation methods [63].

By fluorescently labeled specific antibody against CD45 for leukocytes and cytokeratin 8, 18, 19 for epithelial cells, they found a predictive and independent significance of the number of CTC for overall survival with 177 enrolled patients with various cell types and progression stages [64]. Epithelial cell rarely circulate in healthy women with mean being 0.1 ± 0.2 per 7.5 ml of whole blood.

Approximately 30–40 % of primary breast cancer patients and up to 60 % of metastatic breast cancer patients have CTC. EpCAM serves as a hemophilic adhesion molecule in physiological epithelial organs. Immunopathological analysis of 1715 breast cancer patients revealed that its overexpression was observed in roughly 40 %, and no expression in 10 % [65]. This suggests that the EpCAM-based detection method of CTC is impotent for those 10 % of patients and EpCAM-independent density centrifugation method is being tried to reevaluate those potentially missed population.

However, the story is not so simple. Unlike leukemic cells of mesenchymal origin, carcinoma cells of epithelial origin need to convert themselves to acquire mesenchymal features (expression of vimentin, smooth muscle actin, N-cadherin, etc.) by losing intercellular adhesion (loss of E-cadherin) to shed themselves to make an entry into circulation and convert back to epithelial cells when resettled in metastatic tissues. A report of immune-pathological analysis of 491 breast cancers

showed that the EMT markers are related to basal-type molecular group [66]. This interconversion process is termed epithelial mesenchymal plasticity (EMP) or transition (EMT) and will be discussed in detail in Chap. 11. EpCAM expression levels in CTC were estimated to be 10 % of those in primary and metastatic breast cancer.

Now, we encounter a big problem. CTC does not necessarily retain its phenotype as found in the primary tumor [67, 68]. Of 66 advanced breast cancer patients, a report showed that 29 % HER2-negative primary cancer had HER2-positive CTC isolated by the immune-magnetic method with anti-EpCAM antibody while 42 % HER2-positive primary cancer had HER2-negative CTC [69]. Another report showed with 78 patients HER2-negative primary had HER2-positive CTC in 32 % [70]. Furthermore, a report showed that CTC change from positive to negative after adjuvant therapy, as measured by mRNA levels of cytokeratin 19, was found in 51 % of 179 patients [71]. However, 22 % of the initially negative 258 patients showed a change from negative to positive. If CTC positivity really represents poor prognosis, some patients may have become worse after adjuvant chemotherapy.

To state on the relationship between the two aspects time and cell type, we return to what we have learned from CML. As in the case of CML HSC, a fraction of breast cancer cells can be subjected to transplantation assays with NOD/SCID mice to test their repopulating ability. A suspension of tumor cells in Matrigel was injected into mammary fat pad. Only 100 cells with CD44⁺CD24⁻Lineage⁻ phenotype could develop tumors in this assay, which could be passaged serially to new mice [72]. The ability to form mammospheres in serum-free conditions is thought to be another functional marker for cancer stem cells, although there are some controversies.

The biological significance of aldehyde dehydrogenase 1 (ALDH1) in breast cancer stem cells has been recognized [73, 74]. The master regulator of EMT Twist can up-regulate expression of CD44, activity of ALDH1, and down-regulate CD24 expression (see EMT in Chap. 11). To complicate the breast cancer story, epithelial cells can be transformed to acquire mesenchymal features to become CTC and eventually to metastasize to other organs. Analysis of 39 metastatic breast cancer patients revealed that 62 % of CTC-positive samples by MUC1- and EpCAM-mediated magnetic isolation also were positive for at least one of the EMT markers, Twist, Akt2, and PI3K α and 69 % for ALDH1 (see Chap. 11 tumor microenvironment section) [73]. Only one patient was triple-negative. This indicates that a large population of CTC has tumor-initiating ability after resettlement in distant organs.

9.8.3.2 Metastasis

Bone and lungs are preferentially affected. It is not rare for patients 20–30 years after successful surgical resection to have recurrence in metastatic organs, such as bone. Depending on which organ or tissue a tumor originates from, it has a diagnostic name with the organ or tissue as an adjective as exemplified in lung tumor, hepatic tumor, renal tumor, and so forth. For metastasis, there is a little

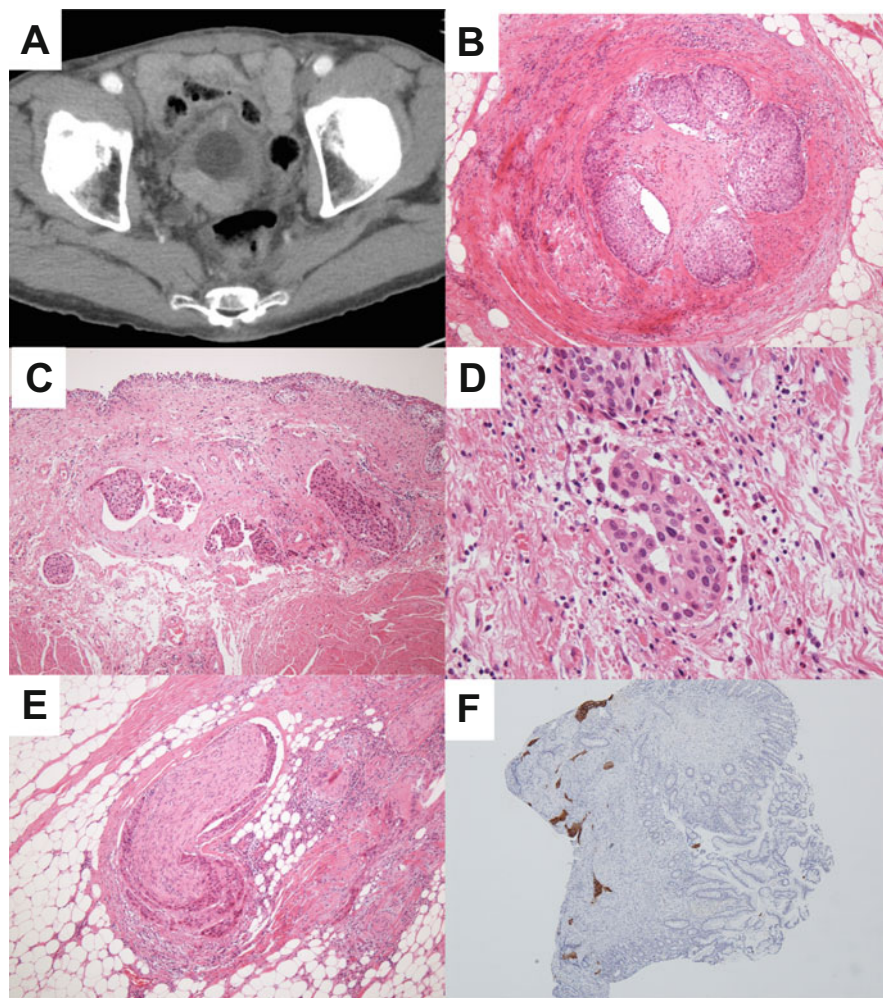


Fig. 9.4 Advanced bladder cancer. A male aged 83 years was hospitalized for renal failure due to bilateral obstruction of vesicoureteral junctions by invasive bladder cancer (A contrast enhancement in CT) (70mm × 38mm × 12mm tumor at triangle, G3, pT3b, ly1, v1, N2, stage IV) and subjected to total cystoprostatectomy with ileal conduit diversion. Pathological examination revealed clustered tumor cell emboli in veins (B) and lymphatic vessels (C, D), and invasion along the neural fibers (E). The patient suffered from rectal bleeding 2 years after the operation and rectal biopsy showed medullary growth of the CK7-positive and CK20-negative tumor cells in submucosal tissues (F), which was compatible to the bladder cancer metastasis. One important point is that hematogenous dissemination does not necessarily results in metastasis in the distant organs. The patient had inguinal lymph node swelling of 2 cm but with no evidence of metastasis in the lungs, brain, and liver until he died of cachexia and severe appetite loss 6 months after the rectal bleeding episode

confusion. While metastatic renal tumor generally means that a renal tumor with a property of metastatic progression to some other organs, metastatic lung tumor sometimes indicates a lung tumor that comes from a primary tumor originated in an extrapulmonary organ.

9.9 Clinical Example of Advanced Bladder Cancer

See Fig. 9.4.

9.10 Concluding Remarks

It is useful to understand cancer from the standpoint of cell type, time, and space. Cell type and genotype of cancer cells may change during progression in which cancer cells can migrate within the body.

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Chapter 10

Basic Research

10.1 Oncogene

10.1.1 *Viral and Cellular Oncogene*

The word <oncogene> sounds a little bit quaint these days. However, we should bear in mind how BCR-ABL was discovered (see Table 9.1 in Chap. 9). The golden marker Ph in CML is indeed the place where the BCR-ABL oncogene is generated. Tumors in animals were known in the past including Rous sarcoma in chicken discovered by Peyton Rous in 1911 that is transmissible from chicken to chicken by RS retrovirus (RSV), Yoshida sarcoma in albino rat discovered by Tomizo Yoshida in 1944 that was chemically induced [1, 2]. Howard Temin showed in 1958 that morphological transformation of chicken fibroblasts by RSV was quantitated in a focus-forming assay, which eventually led to the discovery of reverse transcriptase under his persistent hypothesis that the transformation is mediated by a RSV-dependent DNA intermediate, namely DNA provirus, stabilized in the transformed cells [3]. We should consider the fact that acute viral infection is a self-limited process for propagation of virus but persistent transformation of the infected cells needs to have some stable alterations pertinent to the cells.

Similar retroviruses carrying oncogenic potentials were discovered one after another. Abelson murine leukemia virus (A-MuLV) is one of them, originally isolated from a corticosteroid-treated mouse that was infected with Moloney murine leukemia virus [4]. A-MuLV induces lymphomas in mice in which thymus is spared and can transform B-cell lineage of primary cultured cells as well as oncogene-sensitive NIH3T3 fibroblasts that were spontaneously generated. The viral oncogenes Src and ABL were discovered from RSV and A-MuLV, respectively, and were found to be genetically altered forms of their cellular gene with the same name. Their transduction process resulted in the integration of host genes with activating changes in their virion. The host ABL gene was not only located on the hot spot of human chromosome 9 involved in reciprocal translocation t (9; 22) in

CML but also activated by physical fusion to the BCR gene on chromosome 22. Thus, we have a perfect example of oncogene. The cellular counterpart of the retroviral oncogene viral or v-ABL also is activated as BCR-ABL in CML [5].

Readers should be curious to know how the transforming ABL, whether v-ABL or BCR-ABL, differs from the intact or physiologically expressed ABL. The ABL is a tyrosine kinase enzyme whose activity is regulated intramolecularly as well as by the control of other molecules. The disruption of autoinhibited activity of ABL by deletion of the regulatory SH3 domain in v-ABL or by physical fusion to BCR with the SH3 domain still retained in BCR-ABL resulted in its constitutive enzymatic activation.

As stated in Chap. 9, cancer is understood from three aspects. In this section, in particular, we focus on cell type. Sarcoma cells are those of mesenchymal origin that undergo neoplastic transformation. Therefore, it is reasonable to quantitate transforming potentials of RSV with chicken fibroblasts. Then, what type of cells is suitable to assay oncogenic ability of BCR-ABL? All of us know that HSC should be the most appropriate cell type.

In fact, there are several mouse models in which BCR-ABL was tried to be transduced in HSC.

Retroviral transduction of BCR-ABL in BALB/c mice bone marrow cells and subsequent transplantation into lethally irradiated syngeneic murine recipients induced CML-like myeloproliferative disorders (MPD) with splenomegaly in a mean latent period of 9 weeks [6]. However, other blood disorders, such as ALL, were observed. The BCR-ABL transgenic mouse model utilizing the Tec promoter or inducible one with the tetracycline-controlled transactivator (tTA) under the control of the enhancer element of the murine stem cell leukemia (SCL) gene also resulted in MPD similar to CML [7]. While the Tec tyrosine kinase is activated by RANKL and required for osteoclast differentiation from CFU-GM, its knockout mice displayed B-cell deficiency, suggesting that Tec plays a role in multipotent progenitor cells [8]. The SCL encodes a transcription factor also essential for multipotent progenitor cells.

However, in vitro transformation of Rat1 fibroblasts and bone marrow cells, mainly B cells in Whitlock-Witte long-term bone marrow cultures that support their growth, was conveniently utilized as a useful and reliable assay before the advent of molecular technology to transduce BCR-ABL in HSC. Although both v-ABL and BCR-ABL can transform Rat1 cells and B cells, BCR-ABL fails to transform NIH3T3 cells [9, 10]. The transformation means not only changes in morphology but also growth rate and mode of those indicator cells.

One of the reliable evidence of cell transformation is anchorage-independent three-dimensional growth in soft agar that was documented by Judah Folkman in 1973 [11]. The tested cells included B16 mouse melanoma cells that are frequently used for metastasis experiments and V-79 Chinese hamster lung fibroblasts that were immortalized by a loss of function mutation in p53 (see below).

10.1.2 Signal Transduction-Proliferation

To understand cell growth we need to have ideas on cell signaling and cell cycle. If cells are stimulated with growth factors they start to proliferate actively. The most extensively studied example is epidermal growth factor (EGF) and its receptor (EGFR). Src, ABL, and EGFR, all are tyrosine kinase family of proteins essential for promotion of cell proliferation.

The basic growth-promoting signaling pathway is shown in Fig. 10.1. A growth factor as represented by EGF binds its specific tyrosine kinase receptor EGFR, which induces dimerization of EGFR with acquisition of enhanced enzymatic activity as manifested by auto-phosphorylation. The auto-phosphorylated EGFR not only recruits intracellular signaling molecules, such as Shc, but also phosphorylates them on their tyrosine residues. The sequential molecular interaction, the signaling cascade, is based on the idea that a given molecule has both a sensor domain and an effector domain. To explain the signaling from A1 down to A3, binding of A1 to A2 in the sensor domain changes the conformation of A2, which activates the effector domain to bind A3. The interaction between any two molecules can be binding, enzymatic effect, such as phosphorylation, cleavage, and so forth. The intracellular signaling eventually promotes cell cycle essential for cell proliferation.

BCR-ABL activates at least three signaling pathways for cell proliferation, including the Ras, PI3 kinase, and Stat pathways. Gain of function mutations in EGFR are found in lung cancer and brain tumors. Ras, which comprises at least three main subspecies, H-Ras, K-Ras and N-Ras, is the most frequently, roughly 30%, activated protein by mutation in a variety of human cancers. Therefore, it can be said that mutationally activated Ras is an oncogene.

10.1.3 Cell Cycle

Cell cycle is tightly regulated by cyclin, cyclin-dependent protein kinase (CDK), and their inhibitors consisting of the Cip(p21)/Kip(p27) family and INK4 family [12]. For example, p21 and D1 cyclin promote an assembly of the p21-D1-CDK4/CDK6 (hereafter referred to as CDK4 since both CDK4 and CDK6 behave in a similar fashion) complex in the cytoplasm. When the complex enters into the nucleus, p21 in turn inhibits the kinase activity of CDK4. Although several sets of cyclin-CDK complexes with different molecular structures participate in the transition or during the specific stage of cell cycle, both p21 and p27 generally inhibit their kinase activity. This promiscuous interaction is achieved by the intrinsically disordered nature of p21 and p27 with, for example, the linker helix domain that is stretchable.

The primary function of p27 is to inhibit cyclin E-CDK2 kinase activity [13, 14]. Sequestration of p27 by its recruitment with D-CDK4 complex lets

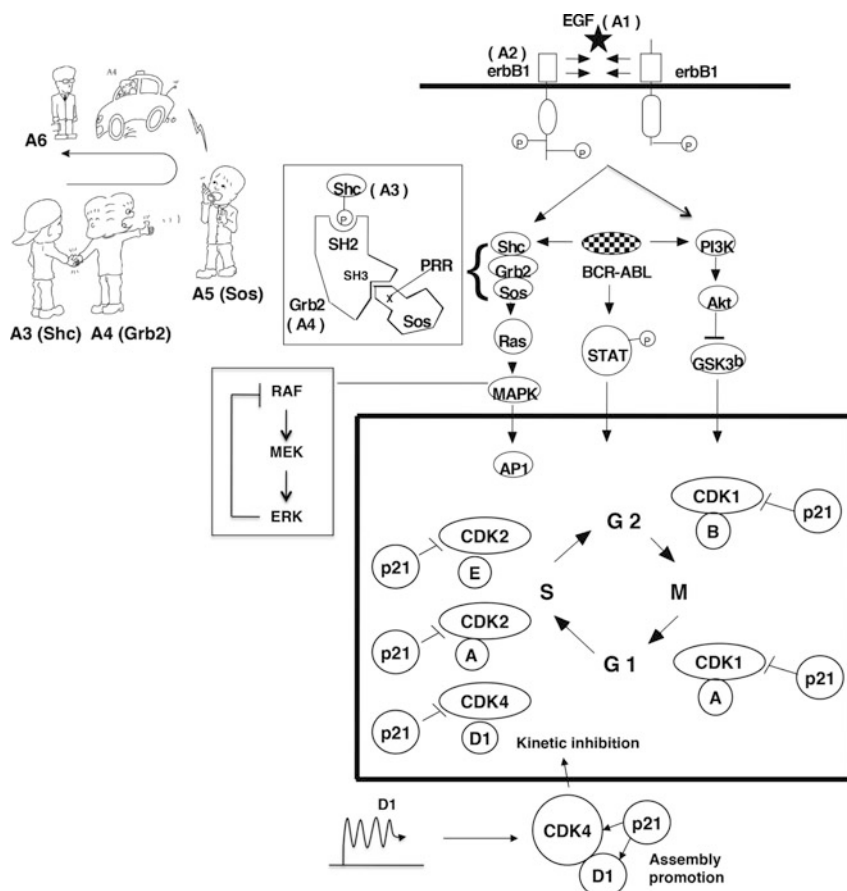


Fig. 10.1 Growth-promoting signaling. The most representative EGF-EGFR signaling is shown. EGFR (also called erbB1) transmits signaling through Shc-Ras and PI3K. The signaling molecule called Grb2 is an adaptor in which tyrosine-phosphorylated Shc binds via its SH2 domain and the proline-rich region (PRR) of GDP-GTP exchanger Sos binds through its SH3 domain. In the cartoon, the Grb2 man (A4) offers his right hand to the A3 (Shc) man by waving his left hand to make a sign to the A5 man with a mobile by which to transmit the information further. The MAPK cascade has a negative feedback from ERK to RAF. In addition to those two pathways, BCR-ABL elicits the third signaling to Stat5. Cell cycle is regulated by cyclins, such as D1 cyclin (D1) and CDKs, whose activity is negatively regulated by cell-cycle inhibitors, including p21

E-CDK2 free from the suppression. However, because p27-D-CDK4 is still active, both CDK4 and CDK2 keep cells proliferating by phosphorylating and thereby inhibiting Rb that blocks S phase entry.

P57Kip2 shares a CDK inhibitory domain with p27 (also called Kip1) and p21 [15]. Expression of p57 in SAOS-2 osteosarcoma cells deficient in both p53 and Rb (see below) resulted in G1 arrest. Down-regulation of p57 often is observed in human cancers.

The INK4 family inhibitors have a narrow specificity for CDKs than the Cip/Kip family [16]. The INK4a locus encodes both p16INK4a and p14 (p19 in mice) ARF (alternative reading frame) that are translated from two alternative reading frames. Whereas p16INK4a inhibits D-CDK4 kinase, p14ARF directly binds Mdm2, which abrogates Mdm2-mediated degradation of p53. Activated Ras induces strong growth-promoting signals but simultaneously stimulates the INK4a – ARF promoters. Collectively, growth-arresting Rb and p53 signals defend cells against growth-promoting Ras-MAPK signals at the level of CDK inhibitors derived from a single locus.

10.1.4 RASopathies

The growth-promoting Ras is active in its GTP-bound form. Due to its intrinsic GTPase activity, GTP-Ras is converted to inactive GDP-Ras. The inactivation step is accelerated by proteins called GTPase-activating protein (GAP)s. Conversely, GDP-Ras is activated by GDP-GTP exchangers, such as SOS1. Activated Ras then initiates the MAPK cascade activation by positively regulating the kinase activity of Raf1 and BRAF, which in turn activate dual specificity kinases MEK1 and MEK2. Both MEKs finally activate ERK1 and ERK2.

Germline mutations in the Ras signaling pathway genes have been found in congenital human disorders, including Noonan syndrome, Costello syndrome, and cardiofaciocutaneous syndrome [17]. The phenotypical similarities are developmental delay and abnormalities in stature, facial feature, and heart. The mutations directly or indirectly activate the Ras pathway, and therefore those syndromes are cancer-prone and referred to as the RASopathies. Based on statistical analysis of 1941 patients, they were found to develop a variety of cancers most frequently in childhood [18]. For example, in Costello syndrome, rhabdomyosarcoma, neuroblastoma, and bladder cancer were reported with the cumulative incidence of 15% by age 20 years. Activating H-Ras mutations at codon 12 that are frequently found as somatic mutations in many sorts of human cancers also are detected in Costello syndrome. Although cardio-facio-cutaneous syndrome has not previously been thought to be cancer-prone, the patients actually have activating mutations in BRAF and MEK with malignancies, including acute lymphoblastic leukemia, non-Hodgkin lymphoma, and rhabdomyosarcoma. MEK mutations are not found in human cancers and mutation sites of BRAF do not necessarily overlap with those in somatic mutations on human cancers. For example, the BRAF mutation V600E, predominant in human various cancers, was seldom found in cardio-facio-cutaneous syndrome. Heterogeneous mutations are found in 70% of the Noonan syndrome patients, including PTPN11, SOS1, K-Ras, and so forth. The most frequent one, PTPN11, encodes SHP2, an SH2 domain-containing nonreceptor tyrosine phosphatase and its mutationally activated forms dephosphorylate p210GAP and Sprouty 1, which eventually leads to Ras activation.

I want to underline two important points. First, a single genetic mutation alone, which includes the activated Ras or BRAF mutation with the most potent transforming ability, is not sufficient to have tumorigenic consequences even if the mutation is transforming when tested in fibroblast transformation assays in vitro (see below for details of assays). Second, cellular protective programs defend cells against the strong growth-promoting Ras signal through the mechanism called oncogene-induced senescence (OIS) (see below).

10.1.5 Endless Division

We have indicator cells, NIH3T3 fibroblasts, and Rat1 fibroblasts to test oncogenic or transforming potentials of candidate proteins. What is needed for readers to enhance understanding of mechanisms of transformation is their historical information as depicted on Rat1 fibroblasts, which requires knowledge on both Ras and p53 (see below). The story started with the discovery of simian virus 40 (SV40) from normal kidney cells of rhesus monkey in 1960 [19]. The virus belongs to polyomavirus with double strand DNAs and can usually be oncogenic for a variety of species except human. The antibody that was spontaneously raised against virus-encoded large T antigen (SV40LT) in SV40-infected and tumor-bearing hamster serum could immunoprecipitate not only SV40LT but also p53 [20]. Mechanistically, SV40LT binds p53 and inactivates its functions, which protects cells against undergoing cell death. SV40LT can immortalize human cells. We have reported immortalization by introduction of SV40LT of rat sinusoidal endothelial cells that are strictly dependent on vascular endothelial growth factor for proliferation but only for just several cell cycles [21].

Primary rat embryonic fibroblasts (REF) prepared from 2-week Fisher 344 rat embryos need to be immortalized first before complete transformation by the activated Ras oncogene, such as H-Ras G12V (Glycine to Valine mutation in the Ras codon 12) initially identified in human bladder cancer [22]. As stated in Chap. 9, p53 mutation could be dominantly oncogenic. In fact, cotransfection of H-Ras G12V and p53 A135V, but not wild-type p53 (-wt), could transform REF [23]. Functional inactivation of p53 by sequestration of p53-wt by SV40LT is equivalent to its physical inactivation by genetic mutations. Rat-1 cells are immortalized REF by SV40LT and therefore susceptible for transformation by H-Ras G12V. Rat-1 transformation by H-Ras G12V is inhibited 25% by superimposed overexpression of p53-wt but enhanced 63% by that of p53 A135V.

10.2 Tumor Suppressor

10.2.1 What Is Tumor Suppressor?

10.2.1.1 Definition of Tumor Suppressor

As explained in Chap. 9, prototypic tumor suppressor genes, such as BRCA1, are recessive in inheritance as a cause of familial cancer syndrome. Inactivation of the two alleles, one germline inherited and the other somatic, is necessary. This is in agreement with the two-hit hypothesis of cancer development. The tumor suppressor genes also are frequently mutated in sporadic cancers. However, molecular analyses of gene products whose functional loss by one-hit appears to expand the concept of tumor suppressor. For example, heterozygous mice lacking p27 develop hyperplasia in various organs and tumors late in life [24]. Fluorescent in situ hybridization (FISH) analysis of 35 primary acute lymphoblastic leukemia patients with chromosome 12p abnormalities showed that hemizygous deletion of p27 located on 12p12 in 29 cases [24]. In multiple endocrine neoplasia (MEN) syndrome type 4, germline p27 deletion mutations have been reported in upstream open reading frame (uORF) of p27 without LOH [14]. Due to the effect of altered uORF on translation during ribosome scanning, expression levels of p27 are reduced. In general, lower the p27 expression levels, poorer the prognosis of cancer patients. Current understanding of the regulation of p27 protein expression levels includes: (1) degradation mediated by Skp2-mediated ubiquitination, (2) cytoplasmic mis-localization after phosphorylation by Akt, and (3) sequestration in the cytoplasm by D-CDK4. The one-hit on the p27 gene gives a similar low dosage influence on its protein expression levels.

Of note, there is some undefined tissue specificity in cancer development in the background of loss of those general cell cycle inhibitors. Mutations of p27 in breast cancer are rare. In familial melanoma patients, germline mutations of p16 are found. Given that melanocytes are supersensitive to UV-induced ROS, cell cycle-independent function of p16 to reduce the ROS levels by unknown mechanisms may play a role in melanoma development apart from desuppression of cell growth.

Another noteworthy issue is that not only oncogenes but also tumor suppressors make a cascade. In the sequence of positive signaling as exemplified by BCR-ABL-Ras-MEK-mediated oncogenic activation, any one of them gives malignant cell transformation with growth promotion [5]. The tumor suppressor Maspin is a serine protease inhibitor and increases cell adhesion to extracellular matrix [25]. Decreased Maspin expression correlates with progression of human prostate cancer. Heterozygous Maspin mice develop hyperplasia in prostate with decreased levels of p27. In short, low dosages of Maspin give rise to low dosages of p27, which forms a tumor suppressor cascade.

Taking tumor suppressor in a broad sense, any genes whose defect contributes to cancer development, directly or indirectly, can be tumor suppressors. In this sense, genes in DNA damage response (DDR) particularly those responsible for sensing

and repair and those in metabolic pathways can be called tumor suppressors. Failed DDR leads to genetic instability that ultimately mutates oncogenes and tumor suppressor genes. Loss of function mutations in metabolic enzymes could support cancer progression even in deficient conditions both in oxygen and nutrients. Thus, mutations take place one after another forming a mutation storm.

Cooperation between oncogene and tumor suppressor has been shown. For example, CML-BC has additional genetic alterations among which those of p53 are most frequent. In fact mating p53+/- and P210BCR-ABL+/- mice displayed more rapid leukemic progression than P210BCR-ABL+/- mice [26]. Transformation of bone marrow cells derived from p53-/- mice (C57BL/6) by BCR-ABL was more pronounced than that of wild-type, and injection of those cells into totally irradiated C57BL-SCID-SzJ immune-deficient mice induced more aggressive leukemic progression [27]. The information supports the idea that p53 loss of function mutation observed in CML-BC can give growth advantages to the CML cells.

10.2.2 DNA Repair System as One Arm in DDR

Human beings have several DNA repair systems to defend the genome against DNA damages caused by a variety of insults including ionizing radiation, DNA-modifying natural and synthetic compounds, reactive oxygen species (ROS) produced by mitochondria, or Nox family of proteins formerly called NADPH oxidase. Here, I briefly describe the essence of molecular biology of the DNA repair machinery.

10.2.2.1 Structural Types of DNA Damages

Ionizing radiation that can introduce double-stranded (ds) DNA breaks induces phosphorylation of histone H2AX on serine 139 which is referred to as γ -component (γ -H2AX) [28]. It was reported that half-maximum γ -H2AX forms in 1 min after irradiation with maximum levels reached by 10 min. Roughly 1% of H2AX is γ -phosphorylated per Gy, which corresponds to 2×10^6 bps per ds break. Detection of γ -H2AX has been conveniently used to show the presence of ds DNA breaks. Then, what are other causes of ds breaks? In addition to ionizing radiation (a), they include (b) replication across a nick during S phase, (c) two close single-stranded breaks by ROS on antiparallel strands, accidental failure of enzymes for physiological ds breaks, such as (d) topoisomerase type II to rejoin the strands, and collateral damage induced by (e) the RAG complex in V(D)J recombination and AID in immunoglobulin class switch in lymphoid cells and (f) mitotic spindle defects.

When the sister chromatid is close to each other during S phase, ds breaks are repaired by homologous recombination (HR). For any phases of cell cycle, nonhomologous DNA end joining (NHEJ) is involved. Both repair systems need

to recognize a diversity of substrate structures with different overhang and DNA ends.

10.2.2.2 HR

The Rad51 recombinase participates in the exchange of the strands [29]. Although human disorders with Rad51 mutations have not been documented, BCR-ABL phosphorylates Rad51 promoting unfaithful HR, which leads to further genetic alterations in CML. BRCA2, which mediates the Rad51 assembly to nucleofilaments, is mutated in the germline of the patients of breast and ovarian cancer syndrome.

10.2.2.3 NHEJ

Double-strand DNA breaks (DSB) by heavy X-ray, for example, are recognized by the MRN complex, consisting of Mre11, Rad50, and NBS1 [30]. The recruited ATM blocks cell cycle by inhibiting CDK activity (the mechanisms involve phosphorylation of the histone variant H2AX, the checkpoint kinase 2-Chk2 and p53) and mediates DNA repair by nonhomologous end joining (NHEJ) program. If the DNA damages are too gross to be repaired, the cells undergo apoptosis. There are two end-joining (EJ) systems for NHEJ for DSB.

The classical one (C-NHEJ) is played by Ku70/80, DNA-PK, Artemis, and DNA ligase IV. Ku recognizes ds DNA breaks with the dissociation constant around 10^{-9} M for DNA ends of any configuration. The Ku recruits 5' endonuclease (the Artemis-DNA-PK complex), polymerase mu, and ligase IV to fix the breaks. The backup EJ (B-NHEJ) is executed by PARP-1 (poly <ADP-ribose> polymerase-1) and ligase III [31]. The B-NHEJ activity is blocked by the DNA-PK-Ku complex before the backup execution of NHEJ is necessary upon the lack of any of the factors in C-NHEJ. At telomeres (see below), TRF2 blocks the DNA-PK-Ku activity to avoid DDR. Therefore, TRF2 deficiency promotes end fusions in a manner dependent on ligase IV and independent of Ku since B-NHEJ takes place to develop end fusions in the absence of Ku that blocks B-NHEJ. In telomerase-deficient cells, DNA-PK and ligase IV are dispensable to achieve end fusions.

Loss-of-function mutations of DNA ligase IV have been reported in humans [32]. The clinical manifestation includes immunodeficiency and developmental retardation resembling Nijmegen breakage syndrome (NBS).

The PARP is activated upon DNA breaks that is sensed by its zinc-finger domain. Chromatin is relaxed by subsequent modification of H1 and H2B by PARP followed by the recruitment of XRCC1 for repair. Inhibitors of PARP, such as DPQ, have additional effects, i.e., antiangiogenic and antimetastatic activity against metastatic melanoma B16F10 cells injected via tail vein by up-regulating VE-cadherin and down-regulating vimentin [33].

10.2.2.4 NER

DNA helix distortions caused by UV, chemicals like cisplatin and heavy ROS are fixed by nucleotide excision repair (NER) [34]. There are two branches: the global genomic repair (GGR) and transcription-coupled repair (TCR). Generally, TCR is faster than GGR.

In GGR, UV-induced cyclobutan pyrimidine dimers (CPDs) and photoproducts (6-4PPs) are sensed by XPE complex (consisting of DDB2 and DDB1) and the XPC-HR23B (human Rad23B) complex, respectively. RPA-XPA also recognizes 6-4PPs and cisplatin lesions. This is followed by the recruitment of TFIIH capable of unwinding the damaged DNAs by two helicases XPB and XPD from the opposite directions. The ERCC1-XPF complex and XPG participate in the 5'- and 3'-incision, respectively. The DNA gaps generated this way are filled by DNA polymerase δ and ϵ and DNA ligase I.

TCR is a complicated process to understand. First, NER factors described above also are engaged in basal transcription. Second, arrested RNA polymerase II makes a stable interaction with CSB, Cockayne syndrome (CS) B protein. CSB is a DNA-dependent ATPase with nucleosome remodeling ability. It should be remembered that the most prominent feature of CS is the inability to restore damaged DNA and resume RNA synthesis. Stabilized CSB near the lesion that stalls RNA pol II recruits NER factors, p300 histone acetyl-transferase (HAT), and the CSA-DDB1-CSN complex. The CSA recruitment to the lesion stimulates further recruitment of HMGN1 (see Chap. 6). Although precise function of HMGN1 remains to be uncovered, it competes with H1 for nucleosomal linkers and HMGN1-KO mice are sensitive to UV-B.

Germline mutations are known for many of the NER factors. The predisposition not only to cancer but also to neuronal disturbances reasonably suggests that the clinical manifestations are due to transcriptional alterations.

10.2.2.5 BER

Modification at single base levels takes place by ROS and chemicals. For example, because 8-oxo-guanine (G) generated by oxidation can make a pair with either C (cytosine) or A (adenine), the oxidation induces transversion of G:C to TA (through replications with 8-oxoG:C to 8-oxoG:A to T:A). The mutagen, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), can yield 3-methyl-A. The 8-oxoG:C and 3-methyl-A:T created this way are recognized by 8-oxo-guanine-DNA glycosylase (OGG1) and 3-methyl-adenine-DNA glycosylase (MPG), respectively, both of which then cleave the linkage between base and sugar to generate apurinic or apyrimidinic (AP) sites. AP endonuclease (APE1) makes a nick at 5' side of the AP site converting the damaged site to single-strand breaks, followed by removal of the sugar left over by DNA polymerase β or FEN1 (FLAP endonuclease) and ligation by combinatory action with DNA ligase III and XRCC1 (X-ray repair

cross-complementing protein-1). Although there have been no solid data to show enhanced BER activity after transcriptional up-regulation of any of the BER genes such as APE1 by genotoxic stress, it should be noted that milieu of persistent inflammation that potentially produces ROS up-regulates APE1. In noncancerous epithelial mucosa of ulcerative colitis, increased APE1 protein level and activity were detected by immune-histochemical analysis and oligonucleotide-base enzymatic assay, respectively, which correlates with the abundance of CD68, a monocyte-macrophage marker. Conversely, approximately 30% of human cancers have somatic mutations in DNA polymerase β . Among others, a seemingly loss of function mutation in germline has been reported in DNA polymerase β at P242R, which is found in 2.4% of human population [35]. In vitro experiments revealed that exogenous expression of the mutant resulted in chromosomal alterations and transformation in mouse embryonic fibroblasts but with no change in mutation frequency and spectrum as monitored by coexpressed lambda genome. When treated with methyl methanesulfonate (MMS), the cells expressing the mutant displayed more breaks in both ss and ds DNAs than the wild-type. Although the ability to bind XRCC1 remains intact, P242R was found to be an enzymatically slower polymerase than the wild-type. Somatic FEN1 loss-of-function mutations also have been reported in various human cancers [36]. The corresponding knock-in mice as represented by the E160D mutation displayed cancers of various cell types and autoimmune disorders with anti-dsDNA antibody, including lung inflammation.

10.2.2.6 Mismatch

The mismatch repair program corrects unpaired bases generated by replication error [37]. The dimeric complex MutS α , consisting of MSH2 and MSH6, recognizes only the mismatched lesion on the newly synthesized daughter strand. MutS α works in concert with the MutL α complex, composed of MLH1 and PMS2, to exert endonuclease activity-mediated removal of the mispaired base.

Loss of the prevention system against replication error can result in mutator phenotypes. In hereditary nonpolyposis colon cancer (HNPCC) (Lynch syndrome), 95% of patients have mutations in two central players MSH2 responsible for recognition and MLH1 that participates in excision of the mismatched DNA lesions [38]. The developed colon cancers bear loss of the second allele. Those are the cardinal features of classical tumor suppressors. Generally, replication errors are frequently found in repeated sequences of the same base called <microsatellite> due to the slippage during replication. In cells without defective mismatch repair system, microsatellite regions either lengthen or shorten, which is called <microsatellite instability> (MSI). The property is applied to clinical examinations of tissue samples to detect MSI by PCR, because MSI in tumor suppressor genes are prone to loss of function mutations.

10.2.3 Linkage to Aneuploidy (see Chap. 12)

Genotoxic stress causing actual DNA damages inhibits transcription and activates ATM and ATR kinases. The MAPK subcascade leads to AP-1 activation and that of Chk1 and Chk2 to p53 up-regulation. AP-1 up-regulates expressions of ERCC1, MSH2, XPF, XPC, etc., and p53 up-regulates those of FEN1, MSH2, DDB2, XPC, etc. [37]. Thus, it is reasonable that UV increases the expression of NER components, such as ERCC1, XPF, DDB2, and XPC, through distinct transcription factors. In addition, ATM-dependent phosphorylation of NEMO (IKK γ) eventually activates NFkB (see Part II for details).

How to connect DDR with aneuploidy that is caused by telomere attrition and abnormal centrosomes is shown below. The centrosome is a multiprotein complex primarily responsible for microtubule organization especially during mitosis in which it also duplicates. For example, astral microtubules released from centrosomes control mitotic spindles. Depletion of centrosomal AKAP450 and pericentrin induces postmitotic cell cycle arrest. We have observed that overexpression of LZTS2, which colocalizes with β -catenin in centrosome and serves as an inhibitor of microtubule-severing protein Katanin, also causes defect in cytokinesis by suppressing microtubule release from centrosomes [39]. An siRNA-mediated knockdown of LZTS2 displayed prominent reduction of central spindle and aster. LZTS2 overexpression in Fps tyrosine kinase-transformed cells showed the presence of micronuclei in 5.6% suggesting mitotic catastrophe, which on addition of caspase inhibitors revealed binucleated cells with extranuclear genome. Human chromosome region 10q23-24 frequently show LOH in prostate cancers and LZTS2 is located in 10q24.3 very close to that of PTEN. In this regard, we proposed that LZTS2 is a tumor suppressor.

Micronuclei that result from abnormal mitosis have nuclear envelopes lacking integrity required to maintain the genomic stability [40]. Isolation of a single chromosome from micronuclei and genomic sequencing as well as live cell imaging techniques revealed that extensive and simultaneous genomic rearrangements, called chromothripsis [41], could take place in one cell division during the cycle progression to S and G2 phase.

Many cancers have a correlation between centrosome abnormality and chromosomal instability (CIN). However, given that those two phenomena often concur in precancerous lesions, such as carcinoma in situ, centrosome defect is an early-stage event in carcinogenesis. Cells with multiple centrosomes seldom undergo multipolar cell division to cell death, but rather they display bipolar division with increased frequency of lagging chromosomes during anaphase. Presence of extra-centrosomes alone was shown to be sufficient to promote chromosome mis-segregation eventually leading to aneuploidy.

10.3 Stabilization of Pathological Proteins

BCR-ABL is a pathological protein that is supposed to be totally absent in physiological conditions, although genetic presence of BCR-ABL mRNAs was reported as mentioned in Chap. 9. The unusual proteins are generally unstable within the cells, because their presence is sensed by molecular chaperones, such as heat shock protein (Hsp) 90 or Hsc70. If they are structurally too unstable to be stabilized by Hsp, they undergo ubiquitination by E3 ubiquitin ligases, such as Cbl and CHIP, and are subjected to proteosomal or lysosomal degradation. We have shown that structurally mature forms of BCR-ABL with the ability to hold ATP as a tyrosine kinase are stabilized by Hsp90 and immature forms immediately after translation by Hsc70 [42]. Therefore, if you inhibit Hsp90 you can see BCR-ABL protein degradation. In many tumor cells in which unknown abnormal proteins are assumed to be expressed, blockade of both Hsp70 and Hsc70 by siRNA-mediated silencing resulted in inhibition of cell growth of several cancer cell lines [43]. A selective Hsp70 inhibitor 2-phenylethynylsulfonamide (PES) also could suppress cell growth of the tumor cell lines [44].

The half-life of p53 in SV40-transformed cells was estimated to be 24 h compared with 20 min in nontransformed cells [45]. Artificially generated p53 by linker insertion mutagenesis at amino acid 158 or 215 exist in two forms, one recruited with Hsc70 and the other without, which can be discriminated by a specific monoclonal antibody called PAb246 [46]. The half-life of the mutated p53-Hsc70 was 150–315 min, whereas that of mutated p53 alone was 15–45 min, giving an apparent half-life of total p53 50–160 min. Cells derived from a methylcholanthrene-transformed fibrosarcoma have elevated protein levels of p53 mutated at C132F with a half-life of 240 min.

Protein folding is mediated by oxidation that forms disulphide bonds. Electron is transferred by endoplasmic reticulum (ER) enzymes, i.e., protein disulphide isomerase and ER oxidoreduction to eventually generate ROS [47]. Conversely, reduction of the disulphide bonds is executed by reduced glutathione (GSH). Therefore, too much protein folding or misfolding is homeostatically controlled by redox programs. UPR (unfolded protein response) activates NFkB. LPS induces ER stress to up-regulate SAP and CRP.

10.4 Cell Death Escape

Oncogene addiction, otherwise cell death.

One of the modes of cell death is apoptosis (see Fig. 2.4 in Chap. 2). Apoptosis is closely related to tumor suppressors. For example, p53 causes cell-cycle arrest. If it passes the point of no return, the cells undergo apoptosis instead of staying senescent at G0 stage of cell cycle by for example DNA damaging reagents.

Apoptosis-inducing genes are in fact activated by p53, including BAX, PUMA, and FAS (see below).

The founder of the apoptosis program is BCL-2, which was originally identified in human follicular B-cell lymphoma in roughly 80% with a chromosomal translocation t (14; 18)(q32; q21), which juxtaposes BCL-2 on chromosome 18 to the transcriptionally active JH region of immunoglobulin heavy chain on chromosome 14, which results in overexpression of BCL-2 in most cases [48]. The genetic rearrangement presumably occurs during IgH variable region recombination in B-cell development in bone marrow.

BCL-2 has four conserved domains called BH (BCL-2 homology) domain 1–4 and transmembrane domain. BCL-2 homologous proteins have been identified that contain one or more of the BH domains [49]. For example, BAD has only the BH3 domain.

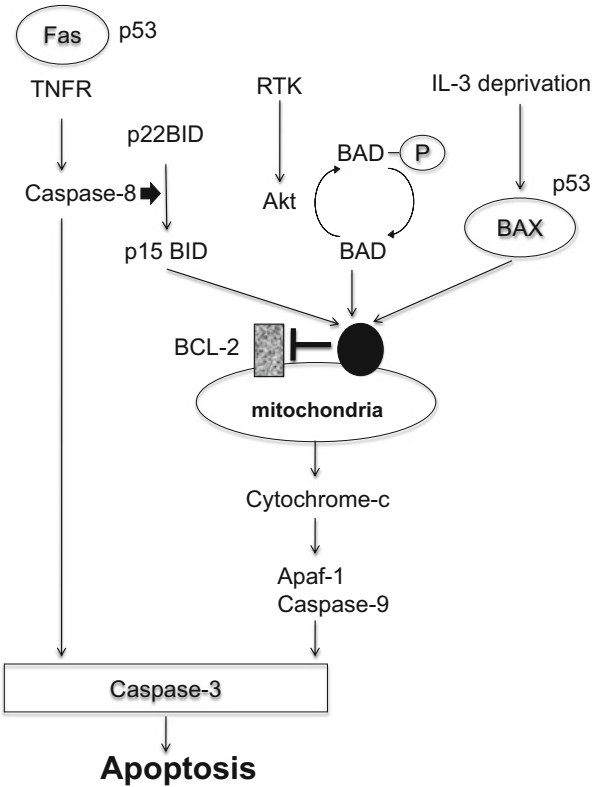
Apoptosis is regulated by the permeability transition pore complex. This machinery consists of BCL-2 and porin (voltage-dependent anion channel or VDAC) in the outer membrane, adenine nucleotide translocase (ANT) in the inner membrane, and cyclophilin D in the matrix [50]. The pore formation leads to rupture of the membrane releasing cytochrome c, which ultimately activates the caspase cascade (Fig. 10.2).

Deprivation of growth factors induces routing of pore-opening protein BAX to the mitochondrial membrane to bind and inhibit BCL-2. Both TNFR and FAS activation cause caspase-8 activation, which cleaves p22BID to p15BID targeting BCL-2 for suppression. BAD also can target BCL-2, but BAD phosphorylation by Akt relocalizes BAD to the cytosol. Those proapoptotic BCL-2 family members, BAX, p15BID, and BAD, release cytochrome c from the mitochondria, which activates the Apaf-1/caspase-9 and subsequent caspase-3 cascade to eventually induce apoptotic cell death. In addition to those mitochondria-dependent and independent pathways, caspase-independent pathways have been reported.

BCR-ABL increases expression of anti-apoptotic protein BCL-2. In Rat1 transformation assay, Rat1 cells can normally grow even in the absence of BCR-ABL. In other words, BCR-ABL is dispensable for growth. If you use Ba/F3 cells, an IL-3-dependent murine Pro-B cell line, as an indicator for transformation, BCR-ABL-transduced Ba/F3 cells can grow faster even in the absence of IL-3 in the culture media than the original non-BCR-ABL-expressing Ba/F3 cells with IL-3. Those BCR-ABL expressers are dependent or addicted to BCR-ABL for their survival and deprivation of BCR-ABL leads to their apoptosis. Both IL-3 and BCR-ABL signal through Stat5 and BCR-ABL can substitute Stat5 signaling that is lost in the absence of IL-3.

Phosphorylated BAD by Akt downstream of BCR-ABL signaling loses its binding affinity and therefore inhibitory ability for BCL-2. BCL-2 released from BAD can now bind and suppress the pore-opening proteins BAK and BAX. Up-regulation of antiapoptotic BCL-2 and inhibition of proapoptotic BAD, both work in concert with each other to exert antiapoptotic effect in BCR-ABL-transformed cells.

Fig. 10.2 BCL-2 signaling. Growth-promoting receptor tyrosine kinase (RTK), such as EGFR activates Akt, which phosphorylates BAD, thereby releasing the BCL-2 inhibition in mitochondria



10.5 Endless Mitosis

Both cell proliferation and senescence are the next subjects. We focus on the endless growth, more precisely how unlimited division of cancer cells is achieved. In addition to the checkpoint at each of the cell-cycle stage, there is a system to monitor the life span of each cell: the telomere system. In contrast to most of the prokaryotic circular DNA, mammalian genome DNA is linear. This causes the so-called <end replication problem>, in which DNA polymerase fails to replicate the chromosome ends every time DNA replicates during the S phase, resulting in the shortening of the ends at a rate of 50–200 bp per cell cycle (average of 77 bp in normal human fibroblasts that lack detectable telomerase activity) [51]. The chromosome DNA ends are called telomeres whose length is 5–15 kb in humans with characteristic conserved tandem repeats of 5'-TTAGGG-3' in both human and mouse, ending in a 3' single-strand overhang TTAGGG repeat of 50–500 nt [52].

To state the conclusion first, telomere structures are recognized as DNA damage unless protected by associated proteins to avoid DNA damage response (DDR). Accumulating evidence shows that telomere-associated proteins regulate cellular aging, some of which are genetically mutated in human diseases. For example,

genetic loss-of-function mutations are found in the WRN DNA helicase gene in the well-known premature aging disorder Werner syndrome with an autosomal recessive trait. Similar mutations have been shown in ATM and NBS1 genes in ataxia-telangiectasia and Nijmegen breakage syndrome, respectively. The common feature shared by the WRN, ATM, and NBS1 proteins is that they bind TRF (TTAGGG repeat binding factor)1 and TRF2, two major components of the Shelterin complex that is associated with a 12–300 b (average 154 b per chromosome end in normal human fibroblasts) distal single-strand end of telomeres to form a high-order structure called T-loop [53].

The attrition of telomeres continues until they reach a critical length when the mitotic clock stops by the activation of NHEJ. The critically eroded telomeres are recognized as double-strand DNA damages, giving rise to either cellular senescence or apoptosis. It should be noted that it is not the average but the shortest length of telomere that is critical [54]. This is called replicative senescence. Senescence is irreversible cell-cycle arrest in normal cells. To say the opposite, T-loop conceals the overhang and protects the chromosomal ends against DDR. Misleading activation causes end-to-end fusion or aneuploidy. To avoid the catastrophe, the multi-subunit telomerase complex essentially consisting of an RNA-dependent DNA polymerase (telomerase reverse transcriptase, TERT) and a template RNA (telomerase RNA, TER) add newly synthesized tandem hexameric repeats to the 3'-overhang. Basically the telomerase activity is absent in normal somatic cells except for germline and stem cells. Heterozygous loss of function mutations of TERT have been documented in human aplastic anemia patients suffering from impaired hematopoiesis with short telomeres [55].

Do the cancer cells survive even when the suffering patient dies? The answer is yes. Although even if cancer cells stop growing, cachectic conditions without tumor growth eventually come because of the production of inflammatory cytokines and growth factors. This will be discussed in Chap. 12. With the above-mentioned fundamental knowledge, let us look at cancer cells with CML cells as an example. The telomerase activity is thought to be reactivated in roughly 80–90% of human cancers. TERT gene transcription is up-regulated by Myc, estrogen, and androgen. A comparative analysis of 123 peripheral leukocyte samples from CML patients with age-matched controls revealed that CML cells have roughly 1-kb shorter telomere length [56]. CML CD34⁺ cells showed 50% increase in the telomerase activity compared with non-CML CD34⁺ cells. Shorter telomere length and an up to 50-fold increase in the telomerase activity in blast crisis patients were reported. Successful imatinib treatment can increase the length of telomeres. Those facts suggest that CML cells addicted to BCR-ABL depend on reactivation of telomerase due to their active cell cycle. Telomerase-deficient mice were engineered by genetic deletion of the mouse telomerase RNA gene (mTR). As expected, mTR-KO mice displayed age-dependent telomere shortening, shortened life span, and increased incidence of spontaneous cancer development. Crossing of mTR-KO mice to Sca1-BCR-ABLp210 transgenic mice in which p210 is expressed under the control of Sca1 promoter to restrict its expression in stem cells resulted in no development of CML [57].

Senescence versus Quiescence

SASP induces inflammation and affects tumor microenvironment, whereas quiescence affects tumor cell dormancy in relapse and metastasis.

Senescent cells are those at G0 stage of cell cycle as exemplified by neurons that achieved terminal differentiation by exiting from the cell-cycle irreversibly. The inability to reenter the cell cycle is now overcome by the technique to generate iPS [58]. Quiescent cells are another type of cells at G0 stage that retain the capacity to make a reentry into the cell cycle under physiological levels of stimuli.

Physiologically two cell types of quiescent cells, hematopoietic stem cells and endothelial cells have been extensively studied. One thing is clear that those cells can hardly be transformed into neoplasm. For example, even in CML, BCR-ABL is present in HSC without giving rise to stem cell leukemia. HSC in CML does not need the oncogene for survival, explaining the resistance against ABL tyrosine kinase inhibitors. However, cancer cells that become quiescent can be reactivated to proliferate after a long period of quiescence resulting in relapse in the primary or nonprimary sites. Therefore, it is important to study when, how, and where cancer cells become quiescent. A similar situation can be found in HSC and presumably CSC. Importantly the mechanistic explanation of their quiescence and reentry into cell cycle requires knowledge of extrinsic factors in the tissue microenvironment and therefore will be provided in Chap. 11.

In contrast to telomere shortening-dependent replicative senescence, senescence that takes place before telomere shortening is mature enough to cause arrest is called premature senescence [59, 60]. Premature senescence can be induced by various conditions independent of telomere length, even in cancer cells: artificial in vitro culture conditions, such as insufficient supply of nutrients, growth factors, and ambient oxygen in cells that require them for growth, activated oncogenes, such as H-Ras V12 (meaning Ras mutated at codon 12), and BRAF E600, which is called oncogene-induced senescence (OIS), loss of tumor suppressors, such as PTEN, and direct DNA damages by ionizing radiation and DNA-damaging antineoplastics (called drug-induced senescence) [61, 62]. In OIS, senescence is interpreted as a defense mechanism against too much cell growth and may be controlled by Hsp72 [63].

Observed biomarkers of senescence includes induction of senescence-associated β -galactosidase (SA- β -GAL) activity possibly due to lysosomal expansion, senescence-associated heterochromatic foci (SAHF) enriched with histone H3 with methylated Lys9 (H3K9Me3) and more recently uncovered senescence-associated distension of satellite (SADS), which is unraveling of compact satellite DNAs more generally observed in a variety of senescent cells from the early stage of senescence induction [64]. I remind you of fundamental information that euchromatin is where gene expression is

(continued)

active and heterochromatin silenced, whereas satellite DNA is originally discriminated from genomic by density-gradient methods and derived from repetitive sequences at centromeres. Skin cells from aged show increased SA- β -GAL activity with p16 expression.

Autophagy, a catabolic process to break down cellular components in lysosome, has a close linkage with senescence. In OIS in human diploid fibroblasts retrovirally infected with H-Ras V12, transient up-regulation followed by down-regulation of activity of both mTORC1 and mTORC2, a key molecule in protein metabolism and negative regulator of autophagy, was observed [65]. Overexpression of ULK3, which is one of the up-regulated autophagy genes in the OIS, induced both autophagy and senescence. Similarly delayed inactivation of mTOR was seen in premature senescence induced by DNA damaging etoposide and γ -irradiation.

Activation of p53, increased expression of CDK inhibitors p16 or p21, positive feed-forward loop between ROS and PKC δ , and DDR constitute molecular basis of senescence. However, OIS by BRAF V600E neither needs p53 nor triggers DDR, suggesting that some but certainly not all OIS has DDR induced by oncogene-stimulated DNA hyper-replication. Both oncogenic mutations H-Ras V12 and BRAF V600E are frequently found in various cancers. However, rare progression to malignant melanoma in human Spitz nevus, benign melanocytic nevus, in which H-Ras V12 is highly expressed and no change or even spontaneous disappearance of human melanocytic nevi with BRAF V600E and up-regulated expression of p16, are well-studied. This raises a fundamental question of how cells protect themselves against oncogene-stimulated endless mitosis by OIS and whether or not the phenomenon depends on cell type [66].

However, some findings suggest that OIS can be reversed by genetic changes in cancer cells per se and presumably by stimulation by surrounding cells. Cells under OIS are metabolically active and produce high levels of ROS and proinflammatory cytokines, such as IL-6 and IL-8, which is called senescence-associated secretory phenotype (SASP), in a manner dependent on persistent DNA damage [67]. A conditional knock-in model of BRAF V600E in melanocytic cells induced benign melanocytic hyperplasia with no progression for up to 20 months and secondary conditional deletion of PTEN resulted in metastatic melanoma within 10 days with 100% penetrance [66]. ROS per se is mutagenic. Both IL-6 and IL-8 are engaged in tumor progression. Although this will be discussed in Chap. 12, I allude to one experiment: coinjection into nude mice of an immortalized but not transformed T29 cell line with chemokine growth-regulated oncogene 1 (Gro-1)-induced senescent fibroblast cell lines promoted tumor growth [68]. The context is cancer cells versus host cells, but if the situation is cancer

(continued)

cells versus cancer cells, this paracrine mechanism may reactivate cell growth.

If the DNA damage is fixed, the cell can restart growth and DDR is transient. However, if it can hardly be fixed, it should lead to persistent DDR resulting in DNA damage-induced senescence. It was reported that this can happen when telomeric DNAs are damaged. It appears that presence of TRF2 close to the damaged lesion in telomere impedes DNA repair. In yeast, telomeric damages have failed recruitment of DNA ligase IV. Thus, as described above, TRF2 protects normal telomeres against DDR, but its presence results in persistent DDR with irreparable and damaged telomeres [69, 70].

10.6 Altered Metabolism

10.6.1 Basic Information on Glucose Metabolism

Anabolism is a process in which simple molecule, such as glucose, is converted to complex ones, such as glycogen. The reverse process is catabolism. Energy inherent in glucose is extracted by the glycolysis pathway (Embden-Meyerhof pathway) in the cytoplasm, in which glucose is converted to pyruvate. This is followed by either of the two pathways dependent on the condition of oxygen supply.

In anaerobic conditions, pyruvate is converted to lactate by LDHA with regeneration of NAD⁺, which is reused to maintain the glycolysis pathway or contributes to fatty acid synthesis. In physiological liver in mice, levels of NAD⁺ and its rate-limiting synthetic enzyme nicotinamide phosphoribosyltransferase (NAMPT) undergo oscillation and regulated by the clock machinery at the level of chromatin modification in transcription by histone acetyltransferase CLOCK and histone deacetylase SIRT1 [71].

Alternatively, it makes an entry into tricarboxylic acid (TCA) cycle (also called citric acid or Krebs cycle) in mitochondria in aerobic conditions. Therefore, the glycolysis pathway is the only emergency pathway upon oxygen deficiency in which 1 mol of glucose can be converted to 2 mol of ATP and 2 mol of lactate (Fig. 10.3). In contrast to the glycolysis pathway, which does not need oxygen, PDH (pyruvate dehydrogenase complex), which fuels TCA cycle with acetyl-CoA (coenzyme A) by conversion from pyruvate, and TCA cycle require oxygen. In aerobic condition, total high-energy phosphate production as designated by ATP mol during the glycolysis, TCA cycle, and subsequent electron transport chain is 38. Switch from anaerobic to aerobic conditions is sensed by cells as high ATP and low AMP, because amount of ATP in cells is much larger than that of AMP (ATP >> ADP >> AMP) and total levels of ATP+ADP+AMP are maintained constant. Low AMP negatively regulates 6-phosphofructo-1-kinase (6PF1K) that converts F6P to F(1, 6)BP, because AMP positively controls 6PF1K. This results in

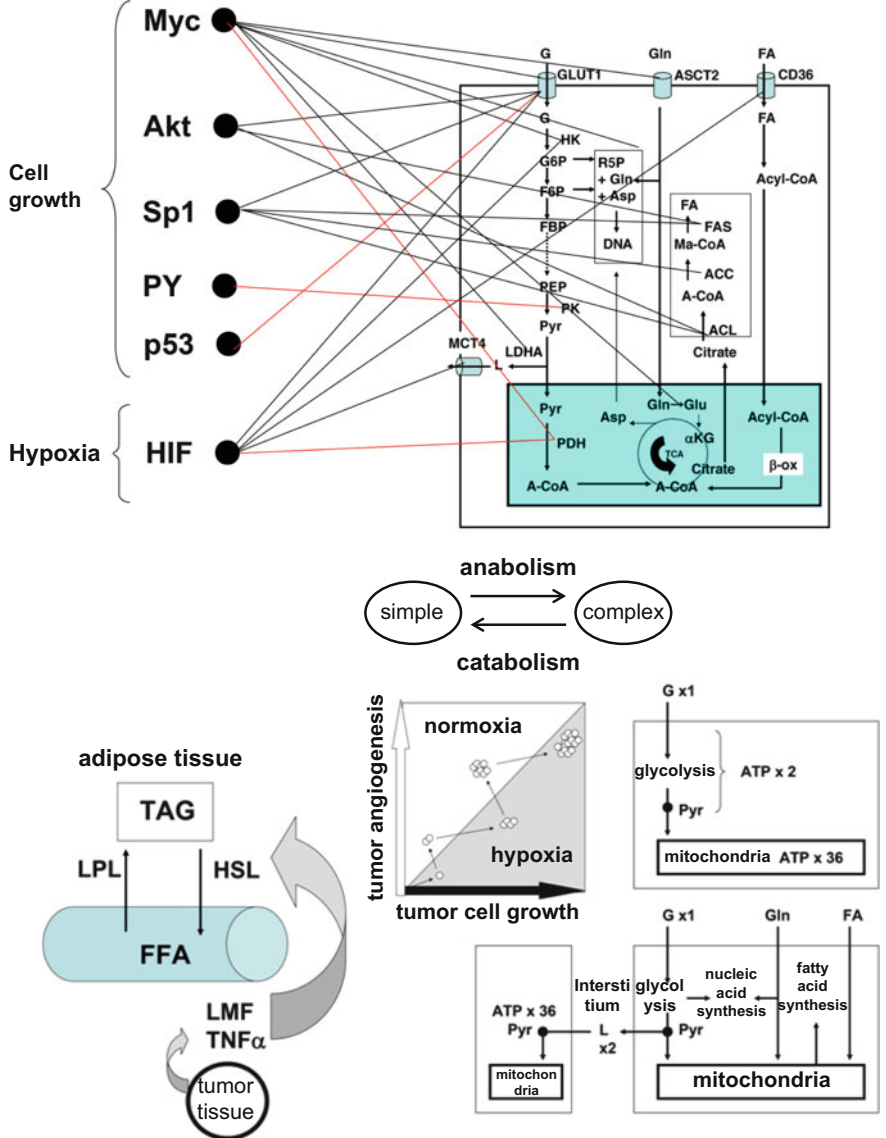


Fig. 10.3 Metabolism in cancer. *Black and red lines* indicate positive and negative signaling, respectively. Activation of tyrosine kinase EGFR phosphorylates tyrosine residues of several proteins (PY) that inhibit the pyruvate kinase M2 isoform (PKM2) by binding its positive allosteric site for fructose 1, 6 bi-phosphate (FBP). TAG triacylglycerol, FFA free fatty acid, LMF lipid-mobilizing factor, HSL hormone-sensitive lipase. LPL lipoprotein lipase. Activation of HSL and tumor-derived LMF are described in Chap. 12 from the standpoint of cancer cachexia. ATP synthesis also is shown

inhibition of the glycolysis pathway, thereby giving low lactate and is called the Pasteur effect. 6PF2K generates F(2, 6)BP from F6P that is a positively allosteric effector of 6PF1K. One of the p53-induced gene products is TIGAR (TP53-induced glycolysis and apoptosis regulator) that has a homology to the fructose-2,6-bisphosphatase [F(2, 6)BPase] domain of the bifunctional kinase and phosphatase 6PF2K/F(2, 6)BPase [72]. Loss of function mutation of p53 results in poor expression of TIGAR, which increases levels of F(2, 6)BP activating 6PFK1 and subsequent glycolysis. Conversely, protective activity of p53 against oxidative stress-induced apoptosis is displayed by TIGAR expression enhancing the pentose phosphate pathway (PPP). A key enzyme in the PPP, G6P dehydrogenase (G6PDH), generates NADPH that serves as a cofactor in the reaction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by glutathione reductase, thereby preventing mutation and apoptosis by neutralization of ROS.

Activation of electron transport chain is perfectly coupled with ATP generation by oxidative phosphorylation. This respiratory control is tightly linked to oxygen and consists of four super-complexes. For example, the complex I comprises more than 40 polypeptide chains and serves as a major source of reactive oxygen species (ROS) from mitochondria and the complex III with 11 subunits contains Rieske iron-sulfur protein responsible for oxidation-reduction reaction [73]. Cytochrome is a heme-bearing protein and classified into a, b, and c depending on the heme type.

Both heart and liver are aerobic organs whose parenchymal cells are enriched in mitochondria. By contrast, red blood cells lack mitochondria and their energy source solely depends on glycolysis.

10.6.2 Principle of Metabolism in Cancer

Generally tumors consist of tumor cells per se and apparently normal stromal cells of host origin. CML bone marrow is not an exception and has both clonal CML cells and stromal cells derived from mesenchymal stem cells. Presence of oncogenes and deficiency of tumor suppressors alter cell metabolism compared with that in normal cells. Because oxygen-dependent functions of mitochondria are crucial in metabolism of mammalian cells, two closely connected issues of nutrition and oxygen states have been extensively studied. During the course of expansion of tumor mass, spatiotemporal nonuniformity is inevitable. Uneven local distribution of tumor growth and tumor vessels takes place at any time points. Wave after wave of growth of certain populations of tumor cells with gene expression, which affects the balance of the glycolysis pathway and mitochondrial functions, occurs at any spatial position within a given tumor tissue. In the waves are cancer cells and normal host cells. Although stromal cells, such as endothelial cells, and fibroblasts have metabolic cell-cell interactions with cancer cells in a paracrine fashion and have even been documented to have some oncogenic mutations in certain conditions, I will discuss the details in Chap. 11. Furthermore, interactions between cancer and other host organs cause ominous cancer cachexia with anorexia and

emancipation due to endocrine-mediated metabolic changes leading to loss of adipose tissue and muscle. I will review the mechanistic information on cachectic mediators in Chap. 12. Here, I focus on just cancer cells.

Both glycolysis and glutaminolysis are altered. In 1956, Otto Warburg claimed that respiration in cancer is irreversibly injured and their mouse ascites tumor cells can obtain energy from anaerobic fermentation [74, 75]. Fermentation is a system by which to convert glucose to lactate in oxygen-deficient conditions. Although peritoneal cavity where their experimental cells grow is hypoxic, cancer cells utilize fermentation even in aerobic condition. He also referred to the experiments by Goldblatt and Cameron in 1953 showing that oxygen deficiency transformed rat cardiac fibroblasts to transplantable cancer cells. The Warburg effect refers to high rate of aerobic fermentation in cancer cells. Usually aerobic conditions inhibit the glycolysis pathway by the Pasteur effect as mentioned above. However, oncoproteins and tumor suppressors not only counteract against the Pasteur effect but also positively control the glycolysis pathway.

What is the advantage of aerobic glycolysis? Although inefficient ATP production as a whole, an increased rate of glycolysis provides a high speed of ATP generation that may surpass that of surrounding host cells. In addition intermediates of the glycolysis pathway can provide tumor cells with substrates for biosynthesis of lipids and nucleic acids absolutely required for cell proliferation. In addition, they also affect mitochondrial complexes and cause reduced rate of respiration. The key points in cancer cells are (1) high ATP consumption and (2) hypoxia.

Cancer cells neither stop respiration nor use only glycolysis pathway. To keep high growth activity with large ATP consumption, increased AMP due to ATP consumption activates 6PF1K in an allosteric manner leading to activation of glycolysis pathway. Hypoxic microenvironment limits but still keeps mitochondrial oxidative phosphorylation. Thus, relative dominance of glycolysis pathway is established. In addition, due to the transcriptional up-regulation of GLUT1 in cancer cells, the rate of glucose uptake is increased by tenfold in the Warburg experiments. Therefore, in a given period of time, cancer cells could produce higher amounts of ATP even from dominant but inefficient glycolysis-mediated synthesis and recessive but efficient respiration-dependent synthesis than normal cells. The speed is the key.

AMPK, a sensor of cellular energy, is activated by binding AMP and promotes the oxidative metabolism (catabolic process) utilized by quiescent cells [76]. AMPK is the only kinase downstream of signaling by LKB1 serine/threonine kinase whose loss-of-function mutation was originally discovered in Peutz-Jeghers syndrome prone to not only colon cancer but also lung and uterine cancers [77]. As a tumor suppressor, AMPK is down-regulated in growing tumors. One revealed mechanism is aberrant expression of E3 ubiquitin ligase MAGE-A3/6-TRIM in tumor cells that ubiquitinates and degrades AMPK α 1, resulting in growth-promoting conditions, such as up-regulation of mTOR and inhibition of autophagy [78]. In addition to the events that take place in a cell-autonomous manner, LKB1-AMPK loss induced CCL2 up-regulation in endometrial cancer cells in a conditional LKB1 inactivation model, in which CCL2 and CCL2-driven macrophages

are required for cancer progression [79]. Conversely, there is a report showing that CCL2 negatively regulated AMPK in prostate cancer cells [80].

Hepatomas express hexokinase II with a lower K_m (0.02 mM) than that of glucokinase (5 mM) in normal hepatocytes. This is another way to shift the balance to glycolysis pathway. Direct binding of hexokinase II porin in mitochondria, which is enhanced by phosphorylation of hexokinase by Akt, not only facilitates the utilization of ATP to phosphorylate glucose but also stabilizes porin to inhibit apoptosis [81].

In general, the oncogene Myc, which can be activated by Ras, directly activates lactate dehydrogenase A (LDHA), glycolysis enzymes, such as hexokinase (HK), glucose transporter GLUT1, and Gln transporter ASCT2. Gln is a nonessential amino acid, but proliferating cancer cells highly need Gln.

Gln not only contributes to the pentose phosphate pathway to generate purines and pyrimidines as a nitrogen donor using the intermediates of glycolysis pathway but also provides Glu with TCA cycle by Gln to Glu conversion by deinhibition of miR-23a/b negatively controlling Glnase, which is followed by the second conversion of Glu to α -KG by glutamate dehydrogenase. Each conversion yields one molecule of ammonia NH_3 . Because high concentrations of ammonia are toxic to cells, cancer cells need to export NH_3 from intracellular to extracellular space. In addition to passive diffusion of gaseous NH_3 across the cell membrane, active transportation is assumed by aquaporin and Rh C glycoprotein, both of which often are expressed in cancer cells [82]. On the other hand, low concentrations approximately 2–4 mM of NH_3 were reported to activate autophagy, thereby protecting tumor cells against $\text{TNF}\alpha$ -induced apoptosis.

GLUT1-mediated glucose addiction in cancer is utilized in clinical imaging examination called positron emission tomography (PET), but it often gives false-negative results in pancreatic cancer. Distinct from the canonical Glu to α -ketoglutarate (α -KG) pathway in TCA cycle, a non-canonical pathway was reported in pancreatic ductal adenocarcinoma (PDAC), in which Gln-derived Asp is transported to cytoplasm followed by its sequential conversion to oxaloacetate (OAA) by Asp transaminase (GOT1), OAA to malate by malate dehydrogenase (MDH1) and malate to pyruvate by malic enzyme (ME1) to eventually generate NADPH [83]. The increased NADPH/NADP⁺ ratio maintains reduced glutathione pools to counteract against ROS. Lentivirus-mediated knockdown of GOT1, MDH1, and ME1 in PDAC 8988T cells reduced the xenograft tumor growth. Importantly, this metabolic reprogramming is dispensable in normal cells but indispensable in PDAC and conducted by oncogenic K-Ras that is mutated in more than 90% of PDAC [84, 85].

Generation of lipids, such as fatty acids (FA), is required for build-up of membrane in actively growing tumor cell without being stored as triglyceride as a consequence. In addition, FA mobilization (see Chap. 12 for discussion on cachexia) from adipose tissues facilitates FA usage by tumor cells. Moreover, tumor cells up-regulate ATP citrate lyase, acetyl-CoA carboxylase-A, and fatty acid synthase—all engaged in generation of FA from citrate (Fig. 10.3).

Here is one example of metabolic study. Metabolome analysis of colon cancer versus noncancer tissues from 16 patients supported the Warburg effect [86]. Low glucose, low pyruvate, high lactate, and high intermediates of the glycolysis pathway, such as G6P, were observed in cancer tissues, which was unrelated to the clinical stages. However, no differences were found in this study in Gln and ATP. In those colon cancer cells, what oncogenes are responsible for the metabolic alterations?

10.6.3 Autophagy

Autophagy regulatory signaling converges on mTOR [87]. Glucose deficiency results in increased AMP/ATP ratio, activating AMPK. Activated AMPK then activates TSC (tuberous sclerosis complex), leading to mTOR inactivation. Hypoxia up-regulates the REDD1 expression (see below), which then stimulates TSC, again leading to mTOR suppression. Starvation of amino acids inhibits mTOR. Those mTOR inhibition cascades stimulate autophagy through ULK-1 (uncoordinated family member [unc]-51-like kinase 1, alternatively called yeast autophagy-related genes (Atg)1 kinase) (Fig. 10.4) complexed to FIP200 (Atg17) and Atg13. The autophagy machine is initiated with this ULK-1 complex followed by participation of three more complexes for elongation process for formation of

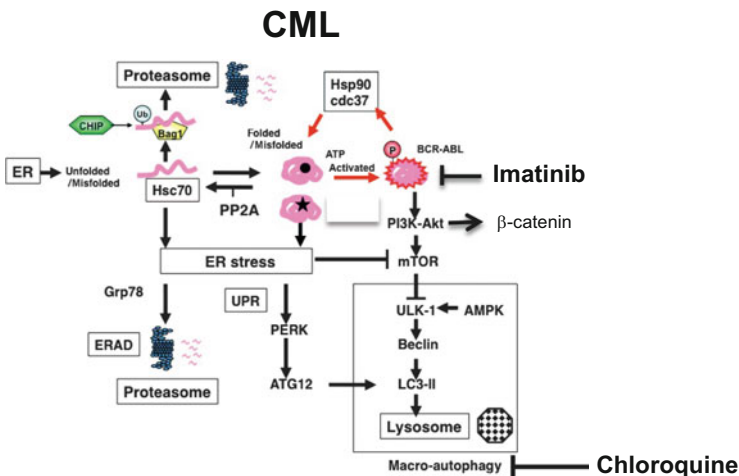


Fig. 10.4 Proteasome, ER stress, and autophagy in CML. In CML, enzymatically mature forms of BCR-ABL can be targeted by imatinib and sudden shut-off of BCR-ABL growth-promoting signaling is adapted by autophagy, which might be inhibited by lysosomotropic agent chloroquine causing lysosomal dilatation and dysfunction. Immature forms of BCR-ABL are chaperoned by Hsc70 after translation and subjected to ubiquitination by CHIP and proteasomal degradation unless properly folded to become mature. Too much protein synthesis, whether mature or immature, causes ER stress, which partly affects the autophagy cascade at LC3-II

autophagosome engulfing cytoplasmic organella and eventually fused with lysosomes. The second is LC3 (ATG8/microtubule-associated protein 1 light chain 3) and ATG12 complexed to ATG5 by ATG7 and ATG10. LC3 is integrated into autophagosome membrane and therefore serves as a reliable marker for autophagy in many experiments in which to monitor extrinsically expressed eGFP-tagged LC3 reporter protein. LC3 is cleaved by ATG4 to LC3-I followed by lipidation to LC3-II, and eventually conjugated to phosphatidylethanolamine.

The difference in LC3-II levels between the presence and absence of bafilomycin A1, an inhibitor of vacuolar-type H^+ -ATPase, shows the amount of LC-II carried over to the lysosomes for degradation, i.e., the autophagic flux. The third is ATG9 and VMP1 interacting with Beclin-1 (ATG6). However, the autophagy program is not so simple. In experiments using MEF doubly deficient in ULK-1 and its homolog ULK-2, it was shown that ULK-1 and -2 are required for autophagy in amino acid depletion but not for deprivation of glucose. Acute deprivation of glucose inactivates mTOR, leading to autophagy, which is restored to an mTOR reactivation state in 24 h. This ULK-1/2-independent autophagy is accompanied by amino acid catabolism with NH_3 generation and NH_3 actually stimulates autophagy in both WT and ULK-1/2-double KO cells [88].

Under noxious conditions for proliferating tumor cells, including nutrient deficiency, hypoxia, oxidative stress, and chemotherapy-induced DNA damage, they <self-eat> (autophagy) or catabolize intracellular macromolecules or organelle, the products of which are recycled for regeneration, and stop proliferating. The activation of autophagy appears to be phase-dependent. When tumor cells initiate proliferation by action of oncogenes, such as Ras, PI3K, and Myc, and/or inactivation of tumor suppressors, such as p53 and PTEN, autophagy is inhibited. However, tumor cells encounter a variety of microenvironmental stress during the course of proliferation. This reactivates autophagy. Given that human breast cancer cells, such as MDA-MB-231, carrying activating mutation of K-Ras failed to grow in soft agar and to maintain glycolysis levels in anti-ATG7 or anti-ATG12 shRNAs, tumor cells appear to hold a baseline level of autophagy activity as a pledge for growth [89]. In other words, autophagy is necessary for tumor growth, although it is suppressed during active proliferation.

Here I highlight two interesting points that are related to autophagy. The first is that Beclin-1 is a tumor suppressor. Monoallelic deletions have been reported in human cancers such as breast cancer and spontaneous cancers developed in mice heterozygously deficient in Beclin-1. In mammosphere-forming human breast cancer stem cells (see Chap. 9), whether from cell lines such as MCF-7 or primary cancer samples, both autophagic flux and Beclin-1 expression are increased. Conversely, shRNA-mediated silencing of Beclin-1 in breast cancer cell lines including MCF-7 cells inhibited self-renewal and xenograft formation by their corresponding mammosphere-forming cells [90]. High levels of autophagy is observed and required for maintenance of stemness of not only breast cancer stem cells as described but also of HSC and MSC. The second point is that cells that fail to utilize autophagy program under noxious conditions may undergo necrosis, which

in turn induces inflammation. To say the opposite, autophagy suppresses inflammation by homeostatic control of metabolic stress.

10.6.4 Oncogene and Oncometabolite

Both dependency on and cooperation with Myc in BCR-ABL-mediated hematopoietic transformation are known. Comparative measurement of the metabolome profile in imatinib-sensitive and -resistant BCR-ABL-expressing cells and imatinib withdrawal experiments to reactivate BCR-ABL, both have revealed that BCR-ABL enhances influxes of glucose and Gln with high concentration of intermediates of glycolysis as well as pentose phosphate pathway, including G6P, F (1, 6)B, PEP, and pyruvate [91].

In addition to metabolic changes enforced by preexisting oncogenes, germline mutations of metabolic enzymes are known to predispose to cancer. Familial cancer syndromes are known in loss of function mutations of fumarate hydratase (FH) and succinate dehydrogenase (SDH), both of which are enzymes of TCA cycle and serve as tumor suppressors [92]. Hereditary pheochromocytoma in SDH and hereditary leiomyomatosis and renal cell cancer in FH are reported. For example, SDH deficiency accumulates succinate, which inhibits PHD (see below), resulting in the stabilization of HIF-1 α [93]. Because this takes place even in normoxic condition, it is called pseudohypoxia.

Aberrant mutations also are found in isocitrate dehydrogenase (IDH) that catalyzes NADP⁺-dependent decarboxylation of isocitrate to yield α -ketoglutarate (α -KG) and NADPH in multiple human cancers, including AML [94] and glioma [95]. IDH1 and IDH2 are localized in peroxisome/cytoplasm and mitochondria, respectively. Mutant IDH converts α -KG to R-2-hydroxyglutarate (2HG), which is an oncometabolite capable of inducing global DNA hypermethylation by competitively inhibiting methylcytosine dioxygenases. The resulting epigenetic alterations are procancerous as confirmed by conditional knock-in mice of IDH1 (R132H) in AML model [94]. An IDH1 inhibitor called AGI-5198 could delay tumor growth in glioma xenograft models with reduction of H3K9 trimethylation in the promoter of astrocyte marker genes, such as GFAP [95].

Another example of metabolic molecule that affects epigenetic regulation of gene expression is butyrate—a short-chain fatty acid derived from bacterial fermentation of dietary fibers in the intestine. As stated in Chap. 5, bacteria-originated vitamin B12 is a non-self but valuable nutrient for host. Butyrate serves not only as an acetyl-group donor for histone acetyltransferases after conversion to acetyl-CoA but also as an HDAC inhibitor. Under suppressed Warburg effect by either low glucose or anti-lactate dehydrogenase siRNA in colon cancer cells, butyrate stimulated cell growth [96]. But when the cells underwent Warburg effect, it showed an inhibitory activity. Given the rather rapid turnover of butyrate (half-life 6 min), suitable drug delivery design is necessary as a pharmaceutical candidate.

Hypoxia (see Fig. 11.3 in Chap. 11 for Hypoxic Tissues)

Hypoxia activates transcription factor HIF-1, a complex of HIF-1 α and HIF-1 β , also called ARNT [97]. Whereas HIF-1 β is constitutively expressed, HIF-1 α levels are regulated in a homeostatic manner. In normoxic condition, hydroxylation at Pro 402 and 564 of HIF-1 α by prolyl hydroxylase (PHD) in molecular oxygen-dependent manner leads to binding of HIF-1 α to VHL, a component of an E3 ubiquitin ligase responsible for HIF-1 α degradation [98]. Deficiency in VHL as observed in renal cell cancer and inhibition of PHD by metabolic intermediates, such as succinate as stated above, cause pseudohypoxia to stabilize HIF-1 α . HIF-1 targets more than 100 genes, including biologically critical ones in tumor biology, such as VEGF, c-Met, fibronectin, GLUT1, LOX, PIGF, CXCL12, and Twist. HIF-1 α inhibits mitochondrial functions, reducing the rate of oxygen consumption.

10.6.5 Mitochondrial Functions**10.6.5.1 Synthesis and Signaling**

Production of ATP by consuming molecular oxygen, sensing of hypoxia, generation of ROS, and triggering apoptosis are the major functions of mitochondria. The organelle consists of outer and inner membranes with intermembrane space in-between. Inside of inner membrane is matrix where mtDNA is present and TCA cycle takes place. MtDNA is the 16.5-kb circular genome present in multicopy and replicates independently of cell cycle-regulated nuclear DNA. The machinery for oxidative phosphorylation (OXPHOS) for the electron transport chain (ETC) after TCA cycle is embedded in the inner membrane and consists of five multiprotein complexes. Electrons generated by oxidation of NADH and FADH₂ by the complex I and II, respectively, are transferred to ubiquinone (coenzyme Q) to form ubiquinol (QH₂) in the complex III. One e⁻ is further passed to Rieske Fe-S protein and eventually to cytochrome c [73]. This reaction generates an intermediate ubisemiquinone. Ubisemiquinone transfers one more e⁻ to cytochrome b, which recycles the e⁻ back to Q or ubisemiquinolone (Q cycle). Because molecular oxygen (O₂) is lipophilic and therefore present in the inner membrane, and electrophilic capable of obtaining e⁻ from ubisemiquinolone, highly reactive ubisemiquinolone can give e⁻ to O₂ to develop superoxide.

While superoxide from the complex I and II are released to the matrix, one from the complex III is to both matrix and intermembrane space. Although levels of superoxide generation are low in physiological conditions, antioxidant defense mechanisms maintain suitable levels of superoxide. Superoxide is converted to hydrogen peroxide H₂O₂ by matrix Mn-SOD and Cu-Zn-SOD present in the intermembrane space. H₂O₂ can pass through the outer membrane to cytosol.

Glutathione peroxidase converts H_2O_2 to H_2O by oxidizing reduced glutathione (GSH) to oxidized form (GSSG). Glutathione reductase recycles GSSG back to GSH in the presence of NADPH supplied by PPP. Both glutathione enzymes exist in mitochondria and cytosol. ETC generates a proton gradient across the inner membrane with low proton in the matrix, which eventually leads to the complex V, ATPase, to produce ATP.

Thus, O_2 consumption and ATP generation couple in mitochondria. However, in hypoxic conditions, O_2 consumption ceases, but ROS generation still occurs or even more abundantly via ubisemiquinolone. It remains to be elucidated how to understand the enzymological paradox of more product (superoxide) from less substrate (molecular oxygen) by the complex III. There should be some mechanisms by which e^- transfer from ubisemiquinolone to molecular oxygen is enhanced in low oxygen.

Mitochondria-derived ROS is necessary for stabilization of HIF-1 α (see below) in hypoxia but not anoxia. Low oxygen concentration is defined as hypoxia between 1 and 2% and anoxia below 0.1%. Cytosol PDH requires O_2 for HIF-1 α hydroxylation at Pro402 and Pro564. When inactivated by the lack of O_2 , failed hydroxylation and subsequent ubiquitination by VHL keep HIF-1 α still undegraded. Inhibition of PDH takes place only when $\text{O}_2 < 5\%$ with maximum inhibition in anoxia. The presumed explanation of inhibitory mechanism is oxidation of Fe^{2+} to Fe^{3+} in the active center of the enzyme. Hydroxylation of Asn at 803 by factor inhibiting HIF (FIH), which is more sensitive to H_2O_2 and therefore could be a sensor for it, also participates in the modulation of HIF-1 α activity [99].

Transcriptional analysis of K-Ras-transformed mouse fibroblasts revealed lower expression of most of the complex I genes and actual decrease of the complex I activity [100]. What will happen when mtDNAs in the complex I are mutated? MtDNA encodes 37 genes, 13 of which are essential polypeptides constituting a part of the OXPHOS enzymes. MtDNA is sensitive to mutations by ROS generated during OXPHOS. MtDNA loss of function mutations accumulate by aging and microinjection of mitochondria from old cells into young cells results in cellular degeneration. Partially defective or destabilized complex I enhance ROS production, which further destabilizes the complex by affecting FeS clusters, etc., making a vicious cycle.

More recently, it has been recognized that mitochondria are signaling organelles in which TCA cycle-mediated, metabolite-producing functions and membrane potential maintenance are coupled. ROS, which can activate HIF-1 α , are exported to the extramitochondrial space, presumably in a manner dependent on the membrane potential. Generated acetyl-CoA is used for acetylation of HDAC epigenetically modulating transcription.

10.6.5.2 From Oncocytoma to Metastatic Tumors by mtDNA Mutations

Oncocytomas are benign tumors of endocrine and exocrine organs with enlarged globular mitochondria. The mitochondrial hyperplasia is thought to be compensatory for an impaired mitochondrial function. They have mtDNA mutations that cause dysfunction and/or disassembly of the complex I consisting of subunits encoded by both mtDNA and nuclear DNA, as represented by mtDNA-encoded MTND1 mutation. Defective ATP synthesis and ROS production are generally observed. An in vitro experiment showed that a loss of function mutation of NADH dehydrogenase subunit 6 resulted in defect of the complex I with overproduction of ROS. This could confer metastatic potentials to tumor cells as tested by cytoplasmic hybrid technology [101].

MtDNA has a high mutation rate, and mutations take place in the germline of female and the somatic form. Germline mutations in ND3 with predisposition to invasive breast cancer and somatic mutations in ND1 in patients of renal cancer have been reported. Inhibition of OXPHOS leads to ROS production.

10.7 Complicated Linkage of Myc and HIF-1 α to Metabolism and DDR

Myc activation is known to promote S phase entry in cell cycle. In addition, Myc when induced by estrogen-inducible system in normal human fibroblasts stimulated production of ROS as judged by dichlorofluorescein diacetate (DCFDA) in G0-G1 without S phase entry [102]. Concomitantly, DNA damages were observed as monitored by Mre11, RPA-70, and γ H2AX, which were not colocalized with p53 and BRCA1. Therefore, both growth-promoting oncogenes, Ras and Myc, stimulate ROS generation, which in turn induce DNA damage without apoptosis. The DNA damage is monitored by the markers, such as Mre11 and γ H2AX, and the DDR certainly lack the direct evidence to show the presence or absence of actually mutated DNAs that lead to tumor progression. Damaged DNAs can be repaired. When tetracycline-regulated conditional Myc expression in human tumor cells in a SCID mice xenograft model system was used, antioxidant NAC inhibited tumor growth by suppressing ROS [103]. However, comparison of the genomes derived from the cells cultured in vitro and xenograft tumors by spectral karyotype analysis, high-density SNP analysis, and comparative genomic hybridization (CGH) analysis revealed no significant difference. Myc-induced ROS makes a major contribution to tumor progression not by further genomic alterations but by ROS-dependent stabilization of HIF-1 α . Thus, oncogene works in concert with hypoxia.

Hypoxia 0.2–1.0% induced twofold increase of ATM activity in a manner independent of DDR as monitored by γ H2AX [104]. The activated ATM-dependent phosphorylation of HIF-1 α at S696 led to transcriptional

activation of genes, including REDD1 (regulated in development and DNA damage response, alternatively called RTP801), GLUT1, and PDK1. REDD1 represses mTOR dependently on Tuberin encoded by the TSC2 gene, a tumor suppressor gene responsible for tuberous sclerosis that manifests benign tumors in various tissues. Thus, hypoxia down-regulates the mTORC1 activity. Because mTORC1 suppresses Ulk-1 and subsequent autophagy, its de inhibition eventually promotes autophagy. In addition, malnutrition activates AMPK, which in turn activates Ulk-1 leading to autophagy. Autophagy then recycles cellular components for energy source.

Oncogenic stress, such as Ras activation, induces cell death and tumor cells need to protect themselves against it in a cell autonomous fashion. Ras up-regulates expression of p62 in an AP-1 enhancer element-mediated manner, which activates NFkB through IKK resulting in induction of ROS scavengers, including ferritin heavy chain [105]. Analysis of human prostate cancer samples revealed down-regulation of p62 in the stromal fibroblasts [106]. Given that p62-KO reduced mTORC, Myc, and glutathione, thereby increasing levels of both ROS and IL-6, it is assumed that inflammatory effects also can take place in a paracrine manner in prostate cancers. NFkB-mediated expression of p62 caused by LPS also mediates up-regulation of p62, which binds Keap 1 de inhibiting Nrf2 transcriptional activity for antioxidant genes [107].

10.8 Breast Cancer

We provide evidence to support what we have learned on breast cancer in clinical settings. Successful treatments have been executed based on tumor cell expression of and dependence on ER and HER2. Therefore, we need to understand why at least those two receptors contribute to cell growth of breast cancer.

10.8.1 *HER2*

Although EGF can induce not only homodimerization of EGFR or HER1 (HER1 is an alternative name of EGFR) but also heterodimerization between HER1 and HER2 followed by their endocytosis, HER2 is a ligand-less receptor and its crystal structure analyses revealed that HER2 has a conformation similar to EGF-bound EGFR even in the absence of EGF [108]. Therefore, HER2 overexpression is sufficient to exert signaling, such as PI3 kinase activation. Approximately 40% of HER2-positive breast cancer patients carry activating mutations in p110 catalytic subunit (PIK3CA) of PI3kinase [109]. Transgenic mice of HER2 under the control of mouse mammary tumor virus (MMTV) promoter harbor mammary tumors, which can be effectively treated by trastuzumab. However, mice with HER2

overexpression and PIK3CA mutation displayed more aggressive breast cancer with metastasis, which was trastuzumab-resistant.

How does trastuzumab inhibit HER2-dependent cell growth? Trastuzumab-bound HER2 is endocytosed and subsequently degraded by proteasome, which results in decreased amount of HER2 on the cell surface. In addition, because trastuzumab is an antibody, antibody-dependent cell-mediated cytotoxicity (ADCC) makes another contribution in a positive attack by immune cells, such as NK cells or macrophages. In fact, its effectiveness on HER2-overexpressing tumors under the host background of deficiency in FcR γ , whose homodimer is coupled to Fc γ RI (CD64) responsible for recognition of Fc portion of IgG, is dramatically abolished (see Chap. 2).

Interestingly, HMGB1 was released under the treatment and the therapeutic effect was abolished in the MyD88-KO background and partially attenuated by anti-HMGB1 antibody. This suggests an involvement of both acquired and innate immunity [110].

10.8.2 Estrogen Receptor

Estrogen stimulates cell growth via ER α , which is a transcription factor that induces expression of cyclin D1 [111, 112]. The elevated levels of D1 promote the assembly of CDK4-D1 that concomitantly recruits p21 in the cytoplasm, sequestering p21 in the nucleus that inhibits the kinase activity of CDK2-cyclinE. Tetracycline-regulated conditional transgenic mice of ER α developed breast cancer with a mean latent period of 11 months with premalignant lesion, such as ductal hyperplasia in 4 months. In human breast cancer cell line MCF7, expression of cyclin G2 is down-regulated by E2 whose receptor ER is recruited to the G2 promoter. Because G2 protects cell cycle against G1-S transition, the deinhibition results in growth stimulation.

As described in Chap. 9, preposition to breast cancer is found in mutations in BRCA1 tumor suppressor. BRCA1-KO cells showed cell death when Nrf2 is diminished. However, estrogen up-regulated Nrf2-dependent expression of antioxidant genes, which promoted the cancer survival [113]. BRCA1-deficient mammary tumor cells transplanted into male mice were stimulated for growth by estrogen administration.

10.8.3 Hormone-Dependent Tumors

We have a chance to think about tumors that are critically dependent on hormones for their growth. The definition of hormone is any molecule that is synthesized in a given organ or tissue and is secreted into circulation to reach different tissues to exert its action through its own receptor. Each process is highly regulated. The most

representative tumors are estrogen-dependent breast and androgen-dependent prostate cancers.

In addition to the ligand-binding domain, receptors for those steroid hormones have the DNA-binding and transcription-activating domains, and directly play a dual, activator, or repressor role in transcription. They are stabilized by recruitment with Hsp90 in the cytoplasm in an inhibited state, and upon binding to estrogen or androgen they enter into the nucleus as an activated homodimer with a variety of cofactors to participate in transcription.

10.9 Prostate Cancer

Because we have learned a lot about breast cancer in Chap. 9, here I will discuss prostate cancer briefly. Androgen transactivates genes involved in lipid and protein synthesis and represses DNA synthesis genes. Expression levels of androgen receptor (AR) are high in luminal epithelial cells but low or absent in basal cells in prostate. Antiandrogen therapy has applied to prostate cancers virtually all of which express androgen receptor (AR) since 70 years ago. Although more than 80% of responses can be initially obtained, they eventually relapse in 2–3 years. Those hormone therapy-resistant prostate cancers are still AR-dependent [114]. For example, even in bone metastasis castration-resistant prostate cancer cells (CRPC) still express AR but with some heterogeneity in its expression level. CTC of prostate cancer also displays heterogeneity in AR expression levels.

Activated AR was shown to down-regulate E-cadherin in breast cancer T47D cells and promoted metastatic dissemination to lung and lymph nodes when injected through the tail vein of NOD/SCID mice that were treated by dihydrotestosterone [115]. In addition, reciprocal activation of AR and Slug, a Snail family transcription factor involved in EMT, is known.

Genetic fusion of AR-regulated TMPRSS2 with ERG is an early event in prostate cancer development and found in both prostatic intraepithelial neoplasia lesions and the adjacent cancer cells or even seemingly healthy individuals [116]. Readers can recall the detection of BCR-ABL gene fusion in normal subjects (see Chap. 9). ERG belongs to an Ets family transcription factor and usually is not significantly expressed in prostatic cells. However, due to the androgen-regulated expression of the fusion partner, TMPRSS2, ERG is expressed in prostatic cancer.

Structurally abnormal fusion gene products often induce ER stress under deprivation of addiction targets: BCR-ABL tyrosine kinase activity in the case of CML and androgen-AR activity in prostate cancer. However, it is not the case in prostate cancer simply because the ERG fusion product goes away under antiandrogen therapy due to its androgen dependency. The ERG fusion gene product activates another transcription factor Sox9, which up-regulates a variety of genes, such as CCL2 and β -catenin [116].

Activating mutations and gene amplification of AR, intratumoral androgen synthesis, expression of MET (HGF receptor) analogous to the circumstance in

breast cancer in which another receptor tyrosine kinase HER2 plays a pivotal role in addition to estrogen, all of these are explanations for the acquisition of resistance in CRPC. Genetic alterations, such as LOH of p53 and PTEN (see Chap. 11), also take place as late events in prostate cancer progression. In the reported PTEN LOH, the tumor-suppressor gene LZTS2, an inhibitor of microtubule-severing protein during cytokinesis, is involved in 13–42% [117]. It safely can be said that CRPC is still addicted to AR but has other genetic alterations independent of AR that facilitate its progression.

10.10 Recapitulation of Li-Fraumeni Syndrome by Mutant Mice

What we have learned in Chap. 9 raises several fundamental questions of why germline mutations of some genes, such as BRCA1 and p53, lead to tumor development. First of all, I state evidence that hereditary disorders can be recapitulated in genetically engineered mice as exemplified by the best-known tumor suppressor gene p53. In a report with analyses of 200 Li-Fraumeni syndrome members, 21% had more than two cancers [118]. I provide a Japanese Li-Fraumeni family with a 38-year-old proband suffering from only gastric cancer with metastatic progression to liver as uncovered at autopsy [119]. A germline missense mutation at codon 273 from CGT (Arg) to CAT (His), R273H, was observed in peripheral blood lymphocytes from the proband and his two children. Both of the gastric tumor and metastatic liver tumor showed p53 overexpression and LOH in the opposite wild-type allele.

In addition to R273H, R172H mutation is observed not only in patients with Li-Fraumeni syndrome but also in many sporadic solid tumors as well as blast crisis in CML. In heterozygous mice with p53R270H/+ or p53 R172H/+ (human codon 273 and 175 correspond to mouse 270 and 172, respectively), carcinomas of lung, liver, skin, kidney, intestinal tract, bone, and B-cell lymphoma developed, most of which were associated with metastatic progression [120, 121]. Although the mean survival time of mice with p53R270H/+, p53 R172H/+, and p53+/- (one allele deletion mutant) were 15.8, 15.2, and 15.4 months, respectively (remember that mean survival time of mice in general is roughly 20–30 months), both p53R270H/+ and p53R172H/+ mice displayed aggressive tumors with metastatic progression as well as premalignant lesions, such as adenomas, whereas p53+/- had low-grade cancers with less frequency (frequency of multiple tumors on necropsy was 44% in p53R270H/+ and 19% in p53+/-). Those murine tumors revealed LOH in 40–66% as frequently found in Li-Fraumeni syndrome. This indicates that both p53R270H and p53R172H have dominant effects on tumorigenesis.

Of note, tumor spectrum varies in p53-deficient mice depending on the strain [122]. For example, more than 60% were malignant lymphoma in the mixed genetic background of 75% C57BL/6 and 25% 129/Sv, whereas teratocarcinoma also was

observed in pure 129/Sv. In p53^{−/−} mice, 75% and 100% develop tumors by 6 and 10 months after birth, respectively.

Different from other tumor suppressor genes, such as BRCA1 and APC, which are certainly inactivated by deletion or truncation, p53 missense mutations in one allele as described above often are found and could result in gain or loss of function of the gene product. The p53 protein is a transcription factor capable of binding DNAs. The DNA-binding domain of p53 is encoded by exon 4–9 in which mutation hotspots predominate including above-mentioned R175 and R273. Those mutations impair the DNA-binding and as a consequence transcription ability of p53 involved in cell-cycle arrest, DNA repair, and apoptosis.

Readers may think that the normal p53 on the opposite allele to the mutated one could function. However, the p53 function depends on its tetramer structure and the mutated p53 serves in a dominant-negative fashion. Thus, to be clear, the p53 missense mutations abrogate its function as a transcription factor, which results in a dominant role in cancer development and progression. If cells fail to arrest cell cycle, repair damaged DNAs, and undergo apoptosis, they are forced to proliferate, acquire mutations, and survive, respectively. Although I do not describe the details of BRCA1 functions, it is assumed to be involved in DNA damage response [123].

10.11 Concluding Remarks

Basic research results provided unusual properties of cancer cells, including oncogenes and tumor suppressors with DNA damage response genes. Pathological proteins are assumed to be stabilized in cancer cells, and altered metabolism is observed for endless mitosis by evading cell death.

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Chapter 11

Tumor Microenvironment

11.1 In the Beginning Were the Tumor Cells

Here I discuss the assumption that tumor cells exist first in the context of host or stromal cells within the restricted tumor tissue.

Components of tumor microenvironment include cells and molecules produced by the cells. The cells can be either residents or guests from other parts of the body, such as bone marrow. The basic discrimination between the two species is whether or not they mostly come in and out of circulation. The most representative residents are tumor cells by themselves and their surrounding fibroblasts, endothelial cells, and macrophages. White blood cells, such as neutrophils, lymphocytes, and monocytes capable of making a conversion to macrophages after they reach tumor tissues are guests that come from bone marrow by stopping by some other organs for so-called education (see Chap. 12 for details). It is known that malignant tumor cells never solely reside in the primary site but extravasate to achieve metastasis in distant organs and return to the primary site, which is called self-seeding. This is the subject discussed in detail in Chap. 12. In this section, I focus on the physical architecture and functional interplays between cells through transfer of active molecules.

11.2 Normal Vessel Architectures

I only focus on microcirculation that is embedded in a certain organ. Normal microvessels form a well-organized branching architecture with bifurcation and terminate as arterioles that are innervated and surrounded by smooth muscle cells and followed by capillaries and postcapillary venules that circulate back the capillary blood contents. Both capillaries and venules lack innervation and smooth

muscle cells but instead wrapped by contractile pericytes (alternatively called mural cells or Rouget cells after the name of the discoverer Charles Rouget) with gaps in-between. Pericytes are embedded within the basement membrane that surrounds the endothelial lining from the abluminal side and regulate vascular patterning by stabilizing endothelial cells and inducing endothelial apoptosis during sprouting angiogenesis. The major basement membrane components include heterotrimeric glycoproteins, such as laminin and type IV collagen, both of which self-polymerize, with another glycoprotein nidogen that interconnects the two to form sheet-like structures of 50–300-nm thick [1]. Given the presence of basement membrane during sprouting, angiogenesis does not necessarily accompany breaching of basement membrane. Opening and closing of interendothelial junctions, which is almost missing in postcapillary venules, and basement membrane integrity regulates the endothelial barrier against blood proteins and cells.

Basically, leukocytes navigate through venules by actively looking for gaps between surrounding pericytes in which expression levels of basement membrane components are low. High-resolution imaging of whole mounted samples under nonstimulated conditions, including mouse ear skin, mesenteric tissue, peritoneal wall, and cremaster muscle, by immunofluorescence staining and three-dimensional analysis, revealed that size of the low basement membrane regions varies up to $192\ \mu\text{m}^2$ [2]. Those regions are not holes and basement membrane components are continuously present. They serve as gates for leukocyte transmigration. A linear positive or reverse correlation between the number of the low basement membrane region and that of the pericyte gaps or vessel size, respectively, was observed. In cremaster muscle samples, application of LTB₄, CXCL1, TNF α , or LPS resulted in enlargement of the low regions by approximately 80% compared with control. Neutrophils enlarge the low expression areas by neutrophil elastase and its pharmacological inhibition results in their transendothelial migration. This is not the case in macrophages.

Lymphatic capillaries that lack pericytes and have discontinuous basement membrane drain excess materials that leak out of the microvessels in a manner of one-way transport ultimately back into blood circulation via thoracic duct. Gaps that totally lack basement membrane are thought to account for up to 30% of the whole vessel area. While junctional complexes in vascular endothelial cells display a zipper-like structure, those in lymphatic endothelial cells show a button-like structure with flaps. By way of precollecting lymphatic vessels, lymphatic capillaries drain into collecting lymphatic vessels that have circumferential smooth muscle cells and valves to ensure unidirectional conduit.

Tissue resident immune cells, such as dendritic cells, also pass through the same lymphatic stream to reach sentinel lymph nodes that are interconnected by collecting lymphatic vessels. By spinning disc confocal imaging study, it was shown that the incomplete basement membrane regions serve as preformed pores for DCs to make an entry, which is followed by their squeezing through flap valves of lymphatic endothelial cells, alternatively by just pushing aside the flap, without remodeling of the basement membrane, such as degradation [3].

With biomarkers for tissue imaging, tissue architectures have been studied extensively. Representative markers include CD31 and VE-cadherin in endothelial cells, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor), and podoplanin in lymphatic endothelial cells, α smooth muscle actin (α -SMA) for vascular smooth muscle cells, α -SMA, desmin, PDGFR- β , and NG2 for pericytes. α -SMA- and PDGFR β -positive cells that are apart from endothelial cells are called stromal myofibroblasts.

11.3 Tumor Vessel Architectures

Tumor consists of tumor cells per se and nontumor cells that are called stromal cells, including endothelial cells, pericytes, myofibroblasts, fibroblasts, macrophages, myeloid cells, and lymphocytes.

Microvessels that are diffusely embedded within intratumoral tissues are dilated, tortuous, and even trifurcated, and branching vessels have uneven diameters. They often lack circumferential pericytes, smooth muscle cells, or basement membrane. Those structural aberrations are observed in both clinical cancers and experimental xenograft tumors. In this case, tumor cells and stromal cells are very close to each other. In some clinical tumors, tumor cell nests that apparently lack endothelial cells are surrounded by well-developed stromal structures that contain majority of blood vessels. Some tumor cells in this type of tumor could be distantly located from stromal cells. An intermediate type between those two extremities also exists.

11.4 Means and Modes of Communication

There are several modes of giving messages from a given cell to surrounding cells by a paracrine fashion. The exchange media can be either free to move to target cells as in the case of diatomic molecules, lipids, peptide growth factors, and microvesicles containing various active molecules, or they can stay in the micro-environment to anchor other cells as exemplified in extracellular matrix (ECM), such as collagen binding to its receptor integrin. Adhesive membrane proteins, such as homophilic E-cadherin and heterophilic Eph-ephrin, can be said to be a special form to send messages to adjacent cells by direct cell-cell contact. In addition to those active messages, passive but still potent messages include those from released intracellular components from necrotic cells and debris of cells or ECM degraded by cells or extrinsic microbes.

11.4.1 Diatomic Molecules

11.4.1.1 O₂: Hypoxia in Tissues

Tumor tissue often is subjected to hypoxia/reoxygenation. One of the essential features of tumor microenvironment is spatiotemporal heterogeneity in both pO₂ and pH. In 1997, the Rakesh K. Jain group applied combined fluorescence ratio imaging microscopy and phosphorescence quenching microscopy techniques to LS174T human colon adenocarcinoma xenografts grown in a transparent dorsal window chamber in SCID mice and revealed that mean values of interstitial pO₂ and pH were 8.3 mmHg and 7.04 in 27-day-old tumors, whereas those in normal subcutaneous tissue were 30 mmHg and 7.3, respectively [4]. Their findings that no correlation between pO₂ and pH was found and that the transvascular pO₂ gradient $\Delta pO_2 = pO_2 \text{ (vessel center)} - pO_2 \text{ (50 } \mu\text{m away from vessel wall)}$ was more prominent in tumor tissue (5.5 mmHg) than in normal subcutaneous tissue (1.9 mmHg) indicate not only structural heterogeneity or abnormality in tumor vasculature but also alterations in metabolism in tumor cells, such as lactate secretion, as described in Chap. 10. No correlation between pO₂ values 50 μm away from the vessel wall and blood flow rate was found. More recently in 2008 after technical progress, by utilizing phosphorescence lifetime imaging analysis in rat R3230 mammary adenocarcinoma grown in window chambers, the Mark W. Dewhirst group showed that up to 50% of vascular pO₂ can change by more than 5 mmHg within 2.5-min interval [5]. The pervasive presence of constantly fluctuating vascular pO₂ indicates that O₂ supply to tumor cells is always changing. Also in 2008, the Kevin M. Brindle group showed by magnetic resonance imaging of pH in vivo by utilizing calculated ratio of H¹³CO₃⁻ and ¹³CO₂ in implanted EL4 mouse lymphoma of 5-mm in size that while the mean intracellular pH was 7.44 the extracellular pH was 6.71 [6].

Let us think of a simple model. When you think of a given tumor cell within the tumor tissue, it is subjected to repeated hypoxia and reoxygenation challenges. When you think of two tumor cells, one hypoxic and the other normoxic, we can assume a symbiotic relationship between the two cells. A hypoxic tumor cell produces lactate by glycolysis and secrete it through monocarboxylate transporter (MCT) 4 that is induced HIF-1 α . The interstitial space becomes acidic, which is a favorable condition for MMP activation for tumor invasion (see below). The released lactate is taken up by MCT1 in the aerobic cells and serves as a substrate for oxidative phosphorylation [7]. MCT1 expression is down-regulated in hypoxic cells.

With O₂ as a substrate, superoxide O₂⁻ is generated by mitochondria as described in Chap. 10 or Nox family of proteins and participates in oxidative stress in the tumor microenvironment.

Other catalytic reactions involved in two-atomic molecules are NO synthase (NOS) generating free radical nitric oxide (nitrogen oxide, nitrogen monoxide, NO) and hemeoxygenase (HO) breaking down heme to biliverdin, iron, and stable

carbon monoxide (CO). As in the case of nonpolar and hydrophobic O₂ and CO₂, both NO and CO are of small polarity and diffuse rather freely across cell membrane to affect the adjacent cells. Both NO and CO lead to cGMP production as a subsequent signal transmitter.

11.4.1.2 NO

For example, NO that is produced by endothelial NOS (eNOS) affects the adjacent vascular smooth muscle cells for relaxation and is highly reactive with a short half-life of a few seconds in circulation. In hypoxia/reoxygenation or reperfusion, NO reacts with O₂⁻ to form strong oxidant peroxynitrite to damage the tissue. NO also activates NFκB.

11.4.1.3 CO Is Anti-inflammatory

CO has been recognized as an environmental pollutant whose exposure in high dosages is lethal due to its suffocating activity by binding to hemoglobin with 200 times higher affinity than O₂. However, it is continuously produced in physiological conditions by HO-1. A reported 6-year-old boy with complete absence of HO-1 displayed growth retardation, anemia, iron deposition, and persistent endothelial damage [8]. Similar manifestations were observed in HO-1 knockout mice with high levels of embryonic lethality [9]. Those characteristics are presumably due not only to lack of physiological CO generation but also inability to dispose and therefore the persistent presence of heme, which eventually causes cytotoxic effects by oxidative cell damages. It also is reported that heme is an endogenous ligand for the TLR4/MD-2 complex [10]. CO, on the other hand, has been reported to disrupt association between TLR4 and MD-2 thereby inhibiting TLR4-activated signaling, such as production of TNFα through NFκB [11, 12]. In addition, a pharmacologically designed CO-releasing molecule (CORM) can activate macrophages to produce anti-inflammatory cytokines, such as IL-10 [13].

However, the relationship between NO and CO is not so simple. NO induces inducible HO (HO-1), which eventually causes production of CO. Therefore, it is understandable to think that NO in the inflammatory phase and CO in the resolution phase [14]. In fact, CD11b-positive cell recruitment in the intratracheal aspiration-induced acid lung injury in 6 h is abrogated by nontoxic levels 500 ppm of CO exposure [15].

Thus, both CO and its generator HO-1 are historically anti-inflammatory. However, elevated HO-1 expression was observed in both freshly isolated hepatocytes and macrophages in adipose tissues from insulin-resistant patients [16]. LPS induces HO-1 expression [17]. NFκB-mediated expression of cytokines, such as IL-6 and TNFα, was blunted in macrophage-specific HO-1-KO cells. Hepatocytes from HO-1-KO mice were hypersensitive to insulin. Thus, it was proposed that

HO-1 brakes or gives a threshold control of the signaling. HO-1-KO increased mitochondrial respiration. However, under a variety of stress, such as H₂O₂ exposure in yeast, hypoxia in skin fibroblasts, and hind limb ischemia in skeletal muscle, HO-1 deficiency decreased mitochondrial respiration (Rolan Stocker, personal communication), suggesting that the effect of HO-1-KO on mitochondrial respiration depends on the context.

Cystathione- β -synthase (CBS) is a sensor for CO, and the enzymatic activity to produce hydrogen sulfide (H₂S) is inhibited by binding CO. An angiogenic role played by H₂S was supported by the impaired vessel formation by CBS silencing in tumor xenografts [18]. CBS also is in charge of protein arginine methylation of phosphofructokinase/fructose biphosphatase type 3 (PFKFB3) by regulating levels of methyl donors, such as S-adenosylmethionine [19]. Methylated and activated PFKFB3 enhances glycolytic flux by increasing F2,6BP, which can activate phosphofructokinase-1 (PFK-1) in an allosteric manner. For tumor cells to tolerate with oxidative stress derived from hypoxia and antineoplastics, HO-1/CO system-mediated inhibition of CBS results in reduced methylation of PFKFB3, making a shift from glycolysis toward the pentose phosphate pathway (PPP). PPP increases levels of NADPH and reduced glutathione to counteract against oxidative stress.

11.4.2 Peptide Growth Factors

Basically, growth of epithelial tumor cells are stimulated by epidermal growth factor (EGF), stromal cells by corresponding factors including endothelial cells by VEGF, pericytes by PDGF, and fibroblasts by FGF.

11.4.3 Lipids: PGE2, LTB4

Synthesis of AA-derived lipids is described in Fig. 1.4 in Chap. 1. COX-2 expression is up-regulated by many cancers.

11.4.4 <Microvesicles> “Black Shuttle Bus”

In tumor microenvironment, both tumor cells and stromal cells generate microvesicles into the extracellular space. The originally found microvesicles from Hodgkin cells from a patient infected with EB virus were similar to what is observed in vesicles for virus budding [20, 21]. Those from tumor cells include exosomes (50–100 nm), apoptotic bodies (~5000 nm), and tumor-derived microvesicles (TMVs) (200–3000 nm). Oncosomes, shedding vesicles, and ectosomes are alternative names to TMVs. Preformed exosomes are gathered in

multivesicular bodies whose fusion with plasma membrane releases the exosomes. Packed in apoptotic bodies that are formed by membrane blebbing are cellular components, such as fragmented DNAs, etc. TMV is formed by outward blebbing. Uptake of both virus particles and tumor cell-derived exosomes is negatively regulated by caveolin-1. In other words, exosome internalization is increased in caveolin-1-KO cells.

Because TMVs are not only free to move in a similar fashion to secreted molecules but also capable of fusion with target cells followed by release of loaded active molecules. What is harbored in TMVs include active EGFR, MMPs (such as MMP-2, MMP-9, and MT1-MMP), FasL, microRNA, and mRNAs [22]. For example, TMVs with activated EGFR target endothelial cells to activate the VEGF-VEGFR2 system, which is similar to tumor-secreted VEGF stimulating endothelial VEGFR [23]. TMVs loaded with MMPs may be able to exert their effects in remote areas to their producer tumor cells. TMVs with FasL can target Fas-expressing activated T cells to induce their apoptosis. Therefore, it is likely that TMVs can transfer the activated signaling affairs of the producers to various types of cells.

Different from exosomes that are released by exocytosis of multivesicular bodies, ectosomes are generated by outward budding at and released directly from the plasma membrane by pinching off. Although their functions may be similar, mechanisms that regulate their production and physiological roles await further investigation [24].

11.4.5 Metabolites

Lactate is the product of the glycolytic pathway in hypoxic conditions as described in Chap. 10, making the intracellular pH around 7.0–7.2. Its export to the extracellular space by monocarboxylate transporter MCT4 contributes to acidification of extracellular pH. Lactate from tumor tissues is imported into endothelial cells via MCT1, which activates NFκB-dependent expression of IL-8.

11.4.6 Contact-Dependent Tunneling Nanotubes

Initially identified in PC 12 neuronal cells, this type of cell-cell communication is mediated by thin plasma membrane structures of 50 nm to over 1 μ meter in diameter and 5–140 μ meter in length. As monitored by cytoplasmic expression of eGFP in endothelial cells and M-orange in mesenchymal stem cell (MSC), not only those artificial imaging molecules but also Mitotracker-visualized mitochondria were transferred to tumor cells, such as human breast cancer MCF7 through this structure [25]. Although biological significance still remains to be established, transfer of active molecules or organelle via tunneling nanotubes from surrounding cells might confer certain activities on tumor cells.

11.5 Tumor Angiogenesis

11.5.1 VEGF

Angiogenesis is a process in which new vessels are generated from the preexisting ones. To achieve the task, endothelial cells need to proliferate, penetrate the basement membrane, and migrate toward the interstitial space by degrading its extracellular matrix. The formation of the invasive endothelial tips, angiogenic sprouting, is followed by stabilization of some and regression of other vessels. This is called vascular remodeling. This multistep process is accomplished by coordinated actions of both proangiogenic and antiangiogenic factors, imbalance of which results in abnormal vascular formation as exemplified by tumor angiogenesis [26].

The most essential growth factor VEGF-A, produced by tumor cells and stromal cells roughly by half and half, induces those morphological changes and also is responsible for enlargement of interendothelial junctions, generation of fenestrations, and vesicovacuolar channels as observed electron-microscopically. All of the abnormalities contribute to enhanced vascular permeability in tumor microenvironment.

Tumor angiogenesis also is observed in blood cell tumors as exemplified by multiple myeloma (see Chap. 9). Both high microvascular density (MVD) and vascular permeability are present in myeloma bone marrow. A cDNA microarray study with 466 samples of bone marrow plasma cells purified by antibody against CD138 (also called syndecan-1, a transmembrane heparin sulfate proteoglycan specifically expressed in plasma cells), including 300 previously untreated myeloma patients, revealed that normal bone marrow plasma cells already express angiogenic factors, such as VEGF and IGF-1 but not bFGF, which was absent in memory B cells. As stated earlier, myeloma cells are generated by multistep carcinogenesis. Aberrant expression of angiogenic factors, including bFGF and HGF, was found after malignant transformation [27]. However, the correlation between MVD and myeloma cell infiltration in the bone marrow are still being debated.

Lymphatic vessels exist both intratumorally and peritumorally with the latter being functional in terms of draining tumor cells. Intratumoral lymphatic vessels are believed to lack draining function, because they are mechanically compressed not by high interstitial pressure but by expanding tumor cell population. The high interstitial pressure, which is thought to be roughly equivalent to the pressure in microvessels in tumor tissues, is caused by both increased vascular permeability in microcirculation and collapse of unidirectional lymphatic drainage. In addition, intratumoral lymphatic vessels seldom have a close connection with α SMA-positive cells. By contrast, peritumoral lymphatic vessels with less pressure from proliferating tumor cells are frequently open, capable of drainage of both fluid and cells including immune and tumor cells.

Dilatation of collecting lymphatic vessels was observed in both experimentally VEGF-D-overexpressing 293EBNA tumors and VEGF-D-positive metastatic

breast cancer patients [28]. Tumor cells producing VEGF-C or VEGF-D, VEGF-D in particular, are close enough to stimulate VEGFR3 to exert signaling in collecting lymphatic vessels. The biological consequences are their dilatation due to increased PGE2 caused by down-regulation of 15-hydroxyprostaglandin dehydrogenase capable of degrading PGE2. Thus, the dilatation is presumed to be not a passive but active process to facilitate lymphatic flow containing fluid as well as metastasizing tumor cells.

11.5.2 VEGF Receptors

VEGF has two receptors, VEGFR1 and VEGFR2, and two coreceptors neuropilin-1 (NP-1) and NP-2, which are both capable of enhancing the VEGFR2 activation through VEGF-dependent interactions with VEGFR2 (Fig. 3.3 in Chap. 3). VEGFR2 is the main receptor for endothelial proliferation. VEGFR1 also is expressed in myeloid cells, such as monocytes and macrophages. Ectopic expression of VEGFRs in tumor cells also has been reported. Notably, coexpression of EGFR and VEGFR1 in tumor cells was shown to promote tumor development [29]. Skin papilloma formation in K5-SOS murine model was impaired in the background of epidermis-specific deletion of either VEGF or VEGFR1. In vitro analysis of the tumor cells revealed that EGF induced VEGFR1 phosphorylation and the VEGF-VEGFR1 autocrine signaling was shown to work in concert with EGF for tumor cell growth.

11.5.3 VEGF and ECM

VEGF165 is cleavable by MMP3, 7, 9, and 19 in the extracellular space at least in vitro, which is inhibited by TIMP1 and 2 [30, 31]. MMPs usually cleave ECM as represented by collagen to which VEGF165 binds through the carboxyl-terminal ECM-binding domain. VEGF cleavage was associated with MMP activity during cell invasion, and cleaved and thereby released VEGF from the ECM induces vascular dilatation contributing to the abnormal tumor vasculature. In vitro studies of VEGF165 premixed with collagen to allow polymerization followed by immersion, namely matrix-bound VEGF165, revealed that lower concentration of matrix-bound VEGF was sufficient to induce VEGFR2 tyrosine phosphorylation at Y1214 for longer period of time than soluble matrix-unbound VEGF165, which led to prolonged endocytosis and p38 activation in a manner dependent on a stable complex formation of VEGFR2 with β 1 integrin. It should be noted that while both β 1 integrin and neuropilin promote VEGFR2 activation, VE-cadherin clustering suppresses it.

11.5.4 Additional Co-receptors for VEGFR

11.5.4.1 CD44

CD44 is a transmembrane receptor with the extracellular portion containing the hyaluronan-binding domain and a variant region and intracellular portion engaged in cytoskeletal activity. Although at least 20 different mRNA species were reported derived from the alternatively spliced variant exon 1–10 (v1–v10) in the variant region (CD44v), the major form of CD44, which is called standard CD44 (CD44s) or hematopoietic CD44 (CD44H), lacks any variant insertions. CD44v is abundantly expressed in many cancers in general and CD44v was reported to interact with a glutamate-cystine transporter xCT, thereby controlling reduced glutathione levels and the redox status [32]. CD44-KO mice displayed defective angiogenesis in implantation of B16 tumor whose growth was reduced and wound closure that was delayed, whereas CD11b⁺ or F4/80⁺ myeloid cell recruitment was unchanged [33]. Also in experimental chronic myeloid leukemia (CML) (see Chap. 9), while transplantation of BCR-ABL-expressing cells in either wild-type or CD44-KO mice gave CML-like phenotypes in 90%, only 13% of the recipients of CD44-deficient BCR-ABL-expressing cells developed the disease, indicating that CD44 is required for engraftment CML-HSC [34]. More specifically, CD44v6 was shown to be expressed and play an angiogenic role in endothelial cells. CD44v6 serves as a co-receptor for VEGFR2 and VEGF165-stimulated activation of Erk was completely abolished by a CD44v6 blocking peptide [35].

11.5.4.2 CD146

Endothelial cells express CD146 that belongs to the Ig superfamily of adhesion molecule. Conditional KO of CD146 showed that VEGF-induced microvessel formation of in vivo Matrigel assay required CD146 that enhanced VEGFR2 phosphorylation [36]. The underlying mechanism is not dependent on VEGF as in the case of NPs. It depends on the binding between CD146 with the Ezrin-Radixin-Moesin (ERM) proteins via the conserved KKGK motif. CD146 lacking the motif impaired endothelial migration without affecting the VEGFR2 phosphorylation. In a xenograft model with human pancreatic cancer cells SW1990 lacking expression of both CD146 and VEGFR2, anti-CD146 blocking antibody suppressed the tumor growth, which was enhanced by combination with anti-VEGF antibody.

11.5.4.3 Ephrin-B2

Ephrin-B2 is co-localized with VEGFR2 in tip cells (see below). VEGF-stimulated filopodial extension in tip cells requires endocytosis of VEGFR2 in an ephrin-B2

reverse signaling-dependent manner. An orthotopic glioma model with mice deficient in ephrin-B2 signaling due to a mutation valine in the cytoplasmic tail critical for PDZ signaling displayed severely impaired tumor growth [37].

11.5.5 Interplay with Growth Factors

11.5.5.1 FGF and PDGF

In a murine fibrosarcoma T241 cell line transfected with FGF2 and/or PDGF-BB, the coexpressed tumors have disorganized vasculature and enhanced lung metastasis [38]. Basically, FGF2 stimulates growth but not cell migration in endothelial cells. While PDGF-BB has an opposite activity, stimulating migration but not proliferation, VEGF does both. However, PDGF-BB is mitogenic and chemotactic for pericytes whose knockout in mice resulted in embryonic lethality due to increased permeability and hemorrhage based on vascular instability. PDGF-BB overexpression alone in tumor cells resulted in poor pericyte recruitment in tumor endothelial cells. Tumor-derived FGF2 up-regulated PDGFR α and β overexpression in endothelial cells giving overresponsiveness to PDGF-BB also derived from tumor cells and PDGF-BB promoted FGFR1 expression in pericytes. The combined effect mimics VEGF overexpression resulting in disorganization of the tumor neovascularization. This raises an important issue for why anti-VEGF therapy alone has been ineffective.

11.5.5.2 TGF β , VEGF-A, and VEGF-C

TGF β R in endothelial cells are TGF β RII and ALK1, knockout mice of either of which exhibited embryonic lethality due to vascular defects [39, 40]. Biological effects of TGF β are rather complicated due to its context dependency. Referring to the simple experiments in vitro, low levels of TGF β ranging from 10 pg/ml to 10 ng/ml in vitro is thought to be inhibitory for endothelial cell migration. TGF β induces expression of critical proteins in angiogenesis, including PDGF-BB, fibronectin, and its receptor integrin α 5 β 1 and collagen IV.

When you closely look at the leading edge of the angiogenic vessels, the dynamic nature of growth factor interplay can be observed. In general, VEGFR3 is exclusively expressed in lymphatic endothelial cells in adults. The exceptions are actively angiogenic endothelial cells in tumors and tip cells. VEGF-A stimulates VEGFR2 on tip cells to induce Notch ligand delta-like 4 (Dll4), which binds Notch-1 on the adjacent stalk cells. The chaperone macrophages positive in Tie2 and F4/80 secrete VEGF-C, which activates the VEGFR3 on the tip cells to reinforce the Notch signaling, down-regulating the expression of VEGFR2 in the stalk cells [41]. TGF β also can directly down-regulate VEGFR2 expression. The combined effect leads to make a brake against the proliferation and migration of stalk cells for

vessel stabilization. Current tip cells are converted to stalk cells under the control of newly generated tip cells. Given that VEGF-C heterozygotes displayed failed sprout fusion, the interplay contributes to ensure anastomosis.

11.5.5.3 GRK2

Ligand binding to GPCR induces its phosphorylation by GRK with recruitment of β -arrestin, which causes endocytosis of the GPCR and subsequent desensitization to the ligand. GRK, especially GRK2, does more than that, phosphorylating other signaling molecules, including peptide growth factor receptors. For example, GRK2 phosphorylates PDGFR to suppress pericyte migration. Importantly, as evidenced by experiments with GRK2+/- mice, its dosage appears to be critical. Implanted melanoma B16F10 tumors in GRK2+/- mice exhibited enhanced growth with poor pericytes and higher infiltration of macrophages [42]. The authors claimed the presence of factors in the tumor-conditioned medium capable of down-regulating endothelial GRK2 expression.

11.5.6 *A General Idea on Coordination with Opposite Activity*

Needless to say VEGF activity (proangiogenic) perfectly depends on itself and VEGFR-mediated signaling. If the inhibitory target of an inhibitor is either VEGF or VEGFR-mediated signaling, the inhibitor can be called anti-VEGF (antangiogenic). In brief, an inhibitor can be a destroyer of dependence. More downstream located the inhibitory target of an inhibitor in VEGFR signaling, it looks more like independent of the VEGF activity. The most important antagonism against the VEGF-VEGFR2 cascade is performed by the soluble form of VEGFR1 (see Fig. 3.3 in Chap. 3). The inhibition also can be execution of an opposite activity. We will discuss the antangiogenesis activity of the prolactin fragment from the standpoint of coagulation in Chap. 16.

11.5.6.1 Maspin

Maspin (mammary serine protease inhibitor) belongs to the serpin (serine protease inhibitor/noninhibitor) superfamily as plasminogen activator inhibitor-1 (PAI-1). Maspin is likely of noninhibitor subgroup due to the absence of the structure essential for targeting proteases. Although predominantly localized in the cytoplasm, maspin also is secreted to bind ECM components or present in the nucleus of cancer cells to exert tumor suppressive effects. Breast cancer patients have better prognosis with stronger or nuclear maspin staining than weak or cytoplasmic

[43]. In this regard, maspin has been assumed to be a tumor suppressor. Although maspin-null mice die around embryonic (E) day 4.5–5.5, the conditional knockout mice in all tissue were viable [44]. Retrovirally transduced maspin expression in human breast cancer cells MDA-MB-231 failed to affect growth on xenograftment in nude mice. In VEGF-stimulated growth of bovine adrenal capillary endothelial cells, an inhibitory effect was observed by maspin with ED50 of 0.2–0.3 μM [45]. The vessel density as judged by CD31 was reduced by 38% in maspin-treated prostate cancer LNCaP xenograft in nude mice. Adenovirus-mediated maspin, but not PAI-1, expression in HUVEC induced apoptosis in vitro, which was rescued by expression of BCL-2, suggesting mitochondria-mediated endothelial cell death [46]. Intracardiac administration of the maspin adenovirus disrupted neovasculature in syngeneic and orthotopic model of TM40D murine mammary tumor cells. Collectively, maspin is not necessary but sufficient for endothelial apoptosis.

11.5.6.2 Angiopoietin

Angiopoietin-1 (Ang1) and angiopoietin-2 can be classified as either angiogenic factors or angiogenesis inhibitors. The main function of angiopoietins is to regulate mural cell attachment for vascular stabilization.

Ang1 produced from mural cells binds Tie2 on cells adjacent to each other, which is called transassociated Tie2. This signals through the Akt-GSK3 β – β catenin pathway to activate transcription of a Notch ligand Dll4 (see below), thereby keeping the endothelial cells in quiescence [47]. In the absence of cell-cell contact mediated by Notch, in which Tie2 is anchored in ECM, the Ang1 signaling cannot induce Dll4 expression.

Ang2 mediates vascular permeability as previously mentioned in Chap. 3, and its high expression is a poor prognostic factor in cancer patients.

11.5.6.3 Vasohibin-1 (EC-Derived)

Vasohibin-1 expression can be induced by VEGF or bFGF in HUVEC but is normally of low levels in endothelial cells. It involves the VEGFR2-PKC δ signaling pathway, which is abrogated by TNF α , IL-1 β , and hypoxia. It does not interfere with VEGFR2 signaling. Enhancement of angiogenesis with immature endothelial cells is observed in LLC tumors transplanted in the hind footpad with increased lung metastasis and inguinal lymph node metastasis in vasohibin-1-KO mice [48]. Because direct injection of LLC through the tail vein failed to make a difference in lung metastasis between WT and KO, the lack of vasohibin-1 in tumor angiogenesis could promote tumor cell entry into the circulation. The enhancement effect is not dramatic in physiological circumstances.

Increased vasohibin-1 expression is reported in human lung cancer and the explanation is that levels of vasohibin-1 expression in patients are not high enough

to overcome the VEGF effect. However, hypoxic tumor regions are expected to lack its expression, which predicts the biological tumor progression phenotypes in vasohibin-1-KO mice.

11.5.6.4 Examples of Antagonism-1: VEGF and CXCL4 (= PF4)

To say the conclusion first, CXCL4 sequesters VEGF and gives CXCR3-B-mediated inhibitory signals independent of VEGFR2 [49]. Since I cannot state all combinations of antagonism and their modes that vary depending on the context, I describe examples of well-known antagonistic factors. For example, VEGF-A is antagonized by CXCL4 (alternatively called platelet factor 4, PF4) (see Chap. 3 for platelet granules). However, even this apparently simple antagonism is actually rather complicated in terms of mechanism.

First of all, let us remember that VEGF-A has four isoforms, VEGF121, 165, 189 and 206, generated by alternative splicing of a single gene, all of which display an antiparallel homodimeric structure. VEGF165 is the most-studied one that contains the heparin-binding domain encoded by its exon7, which is missing in VEGF121. Both VEGF165 and 121 bind and activate VEGFR2, alternatively called KDR, to induce proliferation of endothelial cells through the protein kinase C-Raf-MAPK pathway [50]. Heparin can enhance the mitogenic activity.

Second, CXCL4 is a tetrameric molecule produced in megakaryocytes followed by storage in α -granules in platelets and belongs to the CXC subfamily of chemokines (see Table 2.2 in Chap. 2). CXCL4 binds its seven transmembrane receptor coupled with heterotrimeric G protein CXCR3-B, different from the alternatively spliced CXCR3-A isomer. Through cAMP-PKA and PLC-Ca²⁺ pathways, CXCL4 inhibits endothelial migration and growth. As in the case of VEGF165, CXCL4 binds anticoagulant heparin with high affinity to deprive anti-thrombin III, which depends on heparin for its activity, of heparin to facilitate coagulation at the site of vascular injury. Now we have five components to consider, VEGF-A, VEGFR2, CXCL4, CXCR3-B, and heparin, to discuss on the inhibitory potential of CXCL4 on VEGF-A.

In the absence of heparin, CXCL4 physically binds VEGF165 with high affinity of KD 5 nM but not VEGF121, indicating that CXCL4 binds VEGF via the heparin-binding domain. Proteins with heparin binding domain, such as bFGF and IL-8, are shown to inhibit binding between CXCL4 and VEGF165. Therefore, heparin, but not chondroitin sulfate, by binding and thereby sequestering CXCL4, abrogates the inhibitory effect of CXCL4 on VEGF165-VEFR2 binding, and concomitantly enhances VEGF165-VEGFR2 interaction in a CXCL4-independent manner. In brief, the antagonistic action of CXCL4 on VEGF165 is affected by extracellular matrix component heparin.

Another mechanism was proposed. CXCL4 activates monocytes to produce ROS that causes cell death in endothelial cells in a manner dependent on the contact between the two through β 2 integrin (CD18)-ICAM-1 binding (see Fig. 1.2 in

Chap. 1) [51]. The endothelial regression could be blocked by Nox2 inhibitors, such as diphenyleneiodonium and apocynin.

To further complicate the situation *in vivo*, CXCR3-B signaling upon binding CXCL4 activates μ -calpain that destabilizes integrin β 3 and caspase 3 to cause endothelial regression to limit angiogenesis. The coordination is achieved by the CXCR3-B expression level that is up-regulated in endothelial cells in the restricted period of time 7–21 days after a wound healing model and is absent before (unwounded) and after (perfectly healed) it. To be accurate, CXCR3 has two alternatively spliced variants CXCR3-A and -B. Both bind CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) and CXCL4 binds only CXCR3-B [52]. Both receptors are antiangiogenic. In tumor tissues, CXCR3-A is up-regulated and CXCR3-B is down-regulated.

Of note, CXCL4L1, only identified in primates such as monkey, chimpanzees and human, is derived from duplication of the CXCL4 gene [53]. The subtle changes in amino acid sequence P58L, K66E, and L67H result in decrease in heparin-binding ability and therefore in increase in angiostatic capability. While CXCL4 is stored in α granules in platelet, CXCL4L1 is expressed in tumor cells and smooth muscle cells. Even tumor cells give a negative feedback on tumor angiogenesis and growth. Both CXCL4 and CXCL4L1 attract T cells, DC and NK cells via CXCR3.

11.5.6.5 Examples of Antagonism-2: VEGF and TSP-1 (Thrombospondin-1)

TSP-1 is a homo-trimeric matricellular protein released from platelets, neutrophils, stromal cells, and tumor cells. TSP-1 expression is regulated by Myc and tumor suppressor p53 in a manner dependent on cellular context. For example, in epithelial cells derived from mammary gland or kidney that express SV40 early region protein, hTERT and H-RasV12, the Ras-Myc pathway represses TSP-1 transcription, whereas loss of function of p53 contributes to the repression in transformed fibroblasts [54]. In both cases the activated Ras induces augmented VEGF expression. Like VEGF, it exists in the interstitial space as bound to ECM, such as fibronectin, and is released during acute inflammation to promote the resolution phase. TSP-1 binds the multiligand scavenger receptor CD36 through three TSP1 repeats (3TSR domain), which in turn recruits phosphatase SHP-1 to dephosphorylate Y1175 (Y1173 in mice) essential for mitogenic and morphogenic signaling by VEGFR2 mediated by the recruitment of PLC γ . Conversely, CD36-null mice have augmented tumor angiogenesis and lack dephosphorylation of Y1175. Independently of this mechanism, TSP-1-bound CD36 was reported to activate the Fyn-p38 pathway or up-regulate Fas ligand or TNF α to induce endothelial apoptosis.

Although TSP-1 is thus an endogenous angiogenesis inhibitor, its expression appears to suppress tumor growth at the early stages of tumor progression and tumors eventually escape the suppression by up-regulation of proangiogenic growth factors, such as VEGF, PDGF, Ang2, and bFGF, as judged by tumor implantation

experiments [55]. Overexpression of TSP-1 in murine colon cancer CT26 cells delayed not only primary tumor growth but also liver metastasis after injection through the portal vein but eventually both took place. Crossing MMTV-neu mammary tumor animals with TSP-1-overexpressing transgenic mice also delayed tumor growth by inhibiting MMP9. Conversely, mating with TSP-1-KO revealed significant increases in tumor burden and tumor angiogenesis [56]. Furthermore, TSP-1-KO in the background of p53 deficiency failed to decrease metastatic progression [57]. Also in clinical samples of breast cancer patients, TSP-1 no longer suppressed angiogenesis in metastatic lymph nodes [58].

11.5.6.6 Examples of Antagonism-3: VEGF and Endosialin

In brief, pericyte endosialin binding to endothelial cell-associated basement membrane results in endothelial apoptosis. Endosialin (CD248)-null pericytes can still attach endothelial cells but are defective in selective vessel regression causing enhanced vascular density in a postnatal retina model [59]. Endosialin attenuates VEGF-A-stimulated Y1175 phosphorylation in VEGFR2 and subsequent MAPK activation in HUVEC.

11.5.6.7 Examples of Antagonism-4: VEGF and IFN γ

IFN γ dampens VEGF expression in endothelial cells leading to detrimental effects on them. This explains its antitumor effect by inhibition of tumor angiogenesis (see Chap. 12). In deep vein thrombosis (DVT) samples from autopsy and in mouse DVT model by ligation of inferior vena cava, IFN γ expression was increased in intrathrombotic F4/80⁺ macrophages, which suppressed VEGF expression by the Stat1 pathway [60]. In the background of IFN γ -KO, recanalization within the thrombi was increased.

11.5.6.8 Examples of Antagonism-5: VEGF and Sema3A

The Sema family of proteins originally was identified as transmembrane axon guidance molecules and bind and signal through their transmembrane receptors plexins. This is a counter-receptor system (see Fig. 1.3 in Chap. 1). Exceptionally secreted forms of Semas as represented by Sema3A directly bind coreceptor neuropilins (NP) and signal via plexins. For example, Sema3A binds NP-1. Phenotypes of Sema3A-KO mice suggested that it is involved in maturation of vasculature by antagonizing against VEGF binding VEGFR2 in a NP-1-enhancable manner [61]. In other words, VEGF and Sema3A share a common receptor NP-1. In chorioallantoic membrane (CAM) angiogenesis assay with VEGF165 or bFGF alone or in combination with Sema3A, Sema3A specifically inhibited the VEGF-stimulated vessel growth. Given that VEGF- and bFGF-mediated angiogenesis is

suppressed by inhibition of integrins $\alpha v\beta 5$ and $\alpha v\beta 3$, respectively, and that *Sema3A* inhibits VEGF-induced tyrosine phosphorylation of FAK and Src, both of which are robustly activated by integrin ligation, the inhibitory effect of *Sema3A* is presumably mediated by NP-1 and integrins.

Interestingly, however, vascular permeability with Evans Blue was fivefold stronger in *Sema3A* than VEGF165 at an equivalent molar dosage. *Sema3A* induced tyrosine phosphorylation of VE-cadherin. In tamoxifen-inducible endothelial cell-specific conditional NP-1 KO mice, it was found that NP-1 was required for both VEGF- and *Sema3A*-dependent vascular permeability. Remember that at least three pathways diverge from VEGFR2 activation, i.e., PKC β -ZO-1 (TJ), FAK/Src-PAK (AJ), and PI3K-Akt (AJ) (see Chap. 3). Therefore, a Src inhibitor SKI-606 inhibited VEGF- but not *Sema3A*-stimulated vascular permeability. However, the *Sema3A*-induced permeability was efficiently suppressed by a PI3K γ/δ inhibitor TG100-115.

11.5.7 Linkage Between Vascular Permeability and Intravasation

VEGF-A expression is augmented in tumor tissues causing vascular permeability. As stated in detail in Chap. 3, VEGF-A-activated VEGFR2 leads to activation of Akt, which in turn phosphorylates eNOS releasing the inhibition by caveolin1 and thereby resulting in production of NO. In addition, VEGFR2 induces phosphorylation of VE-cadherin at Y658 and of β -catenin at Y142 disrupting adherens junctions. This facilitates vascular permeability, intravasation of tumor cells, and extravasation of neutrophils in the primary tumor tissue.

Administration of a cell-permeable peptide derived from caveolin-1 capable of binding and inhibiting eNOS reduced the Evans Blue-monitored vascular permeability as well as tumor growth in the subcutaneously implanted LLC in C57BL/6 mice to the levels comparable to those in eNOS-KO mice [62].

When murine ovarian cancer ID-8 cells expressing VEGF-A were plated onto HUVEC monolayers for 6 h, phosphorylation of FAK at Y397 and of VE-cadherin at Y658 took place in an FAK inhibitor PF-271-inhibitable manner. Conditional FAK-inactive knock-in mice showed less lung metastasis with B16F10 melanoma cells [63].

Real-time imaging studies by intravital high-resolution two-photon microscopy in the MMTV-PyMT mouse mammary cancer model revealed that vascular permeability took place in a heterogeneous manner in terms of both space and time and that transendothelial crossing of tumor cells was concurrent with transiently increased vascular permeability mediated by VEGF presumably produced from perivascular Tie2⁺ macrophages [64].

11.6 Blood Lake

Transgenic mice of VEGF with skin-specific expression displayed increased vascular permeability [65]. Conditional transgenic mice of VEGF under the lung-specific club cell secretory protein promoter exhibited pulmonary hemorrhage [66]. Given that tumor cells abundantly produce VEGF, too much VEGF by itself could cause destabilized vessel-mediated tissue hemorrhage. If the subsequent activation of the coagulation system is hampered by any reasons, extravasated blood elements, such as serum factors and blood cells, remain in the space that surrounds the tumor cells. As this space with blood contents becomes larger than tumor cell cluster, the overall picture of the tumor tissue would appear tumor cell islands in the blood pond [67] (Fig. 11.1). The extremes would be leukemic cells in circulation and ascites tumors or peritonitis carcinomatosa, both of which have tumor cells suspended in liquid stroma.

A collection of mouse ascites tumors, including Ehrlich tumor cells, exhibited bloody ascites, high concentrations of tumor cell-derived VEGF in the ascites up to

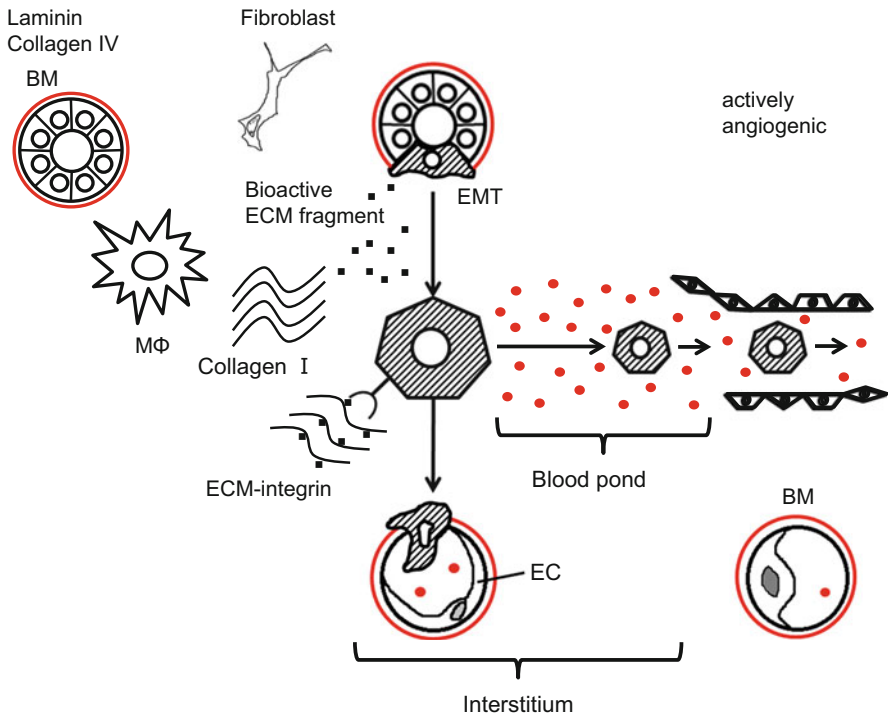


Fig. 11.1 Blood lake. Tumor cells especially after EMT float in the stromal space in which angiogenesis is so active that intravascular elements extravasate. Ascites tumors may be the extremity of blood pond-floating tumor cells

850 ng/ml, and high microvessel densities associated with high perivascular VEGF immune-staining in the peritoneal walls [68].

To introduce this idea of <blood lake>, I briefly refer to the achievements by two Japanese pioneers. One is of hemorrhagic snake venoms by Hideyo Noguchi and the other is Yoshida sarcoma.

11.6.1 Yoshida Sarcoma

At the beginning, I introduce a description of a concept “blood pond” deduced from the experimental results of the Yoshida sarcoma in 1949 [69] (Fig. 11.2).

The vascular system in sarcoma is quite incomplete. The blood that extravasates, a sort of hemorrhage, provides the sarcoma cells with nutrition by running through the interstitial space. If you suppose the most extreme side of stroma of sarcoma, namely the stroma of its own (that develops during the course of sarcoma growth), it is this path of the extravasated blood. If it is true, the stroma of sarcoma is intrinsic to the parenchyma or sarcoma cells and not derived from the normal connective tissues or vessels of host. This situation is particularly evident in transplanted sarcoma, and the essential feature is the same as in the case of primary tumors of spontaneous or experimental occurrence. One interpretation is worth noting. Because stroma of host frequently coexist in the latter case, the stroma of sarcoma is prone to be overlooked (translated by Y. Maru).

Blood, lymphatic fluid, and peritoneal fluid are composed of matrix whose property is liquid and so observed macroscopically. They safely can be said to be liquid tissues. Essentially, malignant tumors belong to the liquid tissue. One could say that their nature is the ability to at least become liquid tissue. The reason that we usually do not see tumors in general as liquid tissue is the presence of stroma derived from host. We should understand that the parenchyma or malignant cells live in the liquid tissue. What I have been describing is the evidence to support the idea. I have tried to strengthen the supportive demonstration by showing a selection of deductive interpretation and physical evidence based on the practical results of histological analysis. I think that at least one working hypothesis would be established (translated by Y. Maru).

Normally, we have a closed circulatory system with capillary between artery and vein except for bone marrow, spleen, and liver, all of which have sinusoid by endothelial cells with fenestration. The microenvironment of the Yoshida sarcoma stated above is similar to the open vascular system found in, for example fly, which is poor in supply of oxygen and nutrition.

「肉腫の血流系統は極めて不完全である。血管外に流出したところの、一種の出血した血液が、実質内の間隙を流れる事によって、栄養が営まれる。肉腫の間質の最も究極的な部分、すなわち固有間質を求めるならば、この流路が、それに当たるわけである。然らばこの固有間質とは、本来実質その者に所属であって、正常組織の血管乃至締結組織に由来するものではない。この事は、移植肉腫において特に明瞭であるが、自然乃至実験的に発生した原発腫瘍に於いても、本質は同様である。ただ後者においては、偶然間質の混在する場合が多いので、本来の者が看過されることが多いのであると解すべきであろう。」

 「血液、淋巴液、腹膜腔液などは、その基質が液体であるから、肉眼的に液体状である。これらの者は、液状の組織と言っていいであろう。悪性腫瘍はその本質において、この意味の液状の組織に属する。少なくとも液状の組織たり得ることを以てその本性とするのではないか。日常遭遇の腫瘍が、一般的には、液状の者としては我々に眼前に現れないのは、偶然間質の存在のためであって、その実質、悪性細胞その者の生態或は生活の場は、液状組織とみるべきではないか。これまで述べてきたのは、このような想定 of 裏付けであった。推理的な考察、実際の組織学的所見等から、選び出したものを整理して、この裏づけを強固にしようとする試みであった。私は、これを以て、少なくとも一つの作業仮説は成り立つだろうと考えている。」

Fig. 11.2 Original writing of Yoshida sarcoma. See English translation in the text

11.6.2 Hideyo Noguchi

11.6.2.1 Hemorrhagic Snake Venoms

Snake Venoms Are Exogenous “Blood Lake”-Makers

Hemorrhagic snake venom causes not only local bleeding at the site of envenomation in many instances but also occasional systemic hemorrhage in distant organs, such as lung and brain. We can find detailed description on biological effects of hemorrhagic snake venoms in the well-known masterpiece called <Snake venom> by Hideyo Noguchi in 1909 [70].

The victims usually die of severe hemorrhage. If snake venom from *Crotalus adamanteus* is injected into the peritoneal cavity of rabbit, extravasation (transendothelial movement of cells from the luminal side to the abluminal side) of

red blood cells takes place within 30 min. Hideyo Noguchi referred to the observation that it consists of two coincidental events that are most likely confused but certainly independent: hemorrhagic and edematous. The former is accompanied by passive extravasation of red blood cells, because they usually lack susceptibility to chemoattractants to acquire cytoskeletal activation leading to active cell migration. The latter represents transendothelial movement of small molecules, such as albumin, whose size is estimated to be approximately 3.5 nm, through endothelial openings. Although it is a famous story that Hideyo Noguchi tried to find out yellow fever virus without electron microscope at that time, what he observed with his optical microscope in the snake venom injection experiments was relatively gross destructions in vessels. Recent understanding is that the edematous region is caused by permeability factors (see Chap. 3) and therefore is frequently abundant in leukocytes.

Snake bite is a physical tissue injury by fangs that causes inflammatory responses. The inflammation should be separated into at least two parts, sterile inflammation as represented by wound healing (see Chap. 1) and inflammation induced by venoms. I mean “sterile” by the absence of microbes. Therefore, snake venom-induced inflammation is caused by both physical and chemical damages. Here, I focus on the latter, especially from the standpoint of coagulation, because we have learned a number of lessons from snakes on the homeostasis of endothelial barrier and clot formation system. Snake venoms contain many active proteins, including proteases and permeability factors.

For example, the *Trimeresurus flavoviridis* habu snake venom contains Tfsv-VEGF that is 52% identical to human VEGF at amino acid levels. Different from VEGF, Tfsv-VEGF had less mitogenic ability but bound VEGFR1 with high affinity to induce vascular permeability, which was abrogated in VEGFR1-TK-KO mice in which the tyrosine kinase domain of VEGFR1 had been knocked out [71].

A natural peptide from *Trimeresurus gramineus*, Trigramin, binds integrin IIb–IIIa through its C-terminal RGD sequence thereby inhibiting platelet crosslink mediated by fibrinogen or vWF (see Chap. 3), both of which use the IIb–IIIa integrin receptor [72]. By this mechanism, the venom can inhibit platelet-dependent coagulation. The antagonistic activity against integrin-mediated cell adhesion was proposed to be called <disintegrin> [73]. Generally disintegrin is thought to allow cells to be released from the tissue architecture, i.e., detachment from ECM or adjacent cells and be floating around hemorrhagic areas in the tumor microenvironment.

The first combinatory molecule of <A Disintegrin and Metalloprotease> (ADAM) in mammalian cells was fertilin engaged in the sperm-egg fusion event [74, 75] (Table 11.1). Fertilin α and β are now called ADAM1 and ADAM2, respectively. Although they lack protease activity, their disintegrin domain on the sperm membrane binds $\alpha 9 \beta 1$ oocyte integrin to exert their effect. However, most of the ADAM family of proteins engaged in tumor biology retains the metalloprotease domain. The study of metalloprotease separately began with biochemical research of proteinases for extracellular matrix as represented by collagen. For example, one of the purified zinc-dependent endopeptidases was previously called gelatinase A but is now listed as matrix metalloprotease 2 or MMP2 [96, 97] (Table 11.2).

Table 11.1 ADAM family

ADAMs	Alternative name	Protease activity	Substrates	KO phenotypes	Roles of KO in cancer	Clinical relevance in human cancer	Reference
ADAM1	Fertilin α	–					
ADAM2	Fertilin β	–					
ADAM3	Cyritestin	–					
ADAM4		–					
ADAM5		–					
ADAM6		–					
ADAM7	GP83	–					
ADAM8	CD156	+	c-kit ligand, L-selectin, TNFR, VCAM-1	Defective in osteoclast formation induced by TNF α		Overexpression in poor prognosis	[76]
ADAM9	Meltrin γ	+	HB-EGF, VCAM-1, c-kit ligand, VE-cadherin	No major abnormalities	Melanoma tumor development.	Breast, pancreas, stomach, skin, liver, lung (cell adhesion, invasion, binding to integrins)	[77]
ADAM10	Kuzbanian	+	Ephrin-A5, TNF α , collagen IV, CD44, HB-EGF, notch, VEGFR2, VE-cadherin	Died at E9.5 because of defective in notch signaling (KO)	Knockdown resulted in cell growth (in vitro)	Associations between protein levels of ADAM10 and tumour grade	[78]
ADAM11		–					

ADAM12	Meltrin α	+	HB-EGF, IGFBP-3, IGFBP-5, fibronectin, collagen IV, VE-cadherin		Decrease of metastasis and tumor growth in MMTV-driven breast cancer model mouse	[79]
ADAM13		—				
ADAM14		—				
ADAM15	Metargidin	+	Collagen IV		Decrease of Neovascularization	[80]
ADAM16		—				
ADAM17	TACE	+	TNF α , TGF β , HB-EGF, APP, L-selectin, CD44, notch, CD40, emmprin, ephrin-B4, ICAM-1, IGFR, IL-1R, IL-6R, PECAM, VCAM1	ADAM17 deltaMP mice died between embryonic day 17.5 (E17.5) and the first day after birth. Open eyelid. Attenuated cornea. Loss of conjunctival sac. Enlarged aortic valves	Reduced neovascularization in ADAM17 lacking mice (Tie2-cre)	[81]
ADAM18	ADAM27	—				
ADAM19	Meltrin β	+	c-kit ligand, HB-EGF, TNF α , TRANCE	Ventricular septal defect		[82]
ADAM20		—			No functional in humans	
ADAM21	ADAM31					
ADAM22		—				
ADAM23	MDC-3	—				
ADAM24	Testase 1	—				
ADAM25	Testase 2	—				
ADAM26	Testase 3	—				
ADAM27	ADAM18					

(continued)

Table 11.1 (continued)

ADAMs	Alternative name	Protease activity	Substrates	KO phenotypes	Roles of KO in cancer	Clinical relevance in human cancer	Reference
ADAM28	MDC-L	+	FASL, CD40L, IGFBP-3			In non-small cell lung carcinoma, positive correlation with lymph node metastasis	[83]
ADAM29		–					
ADAM30		+					
ADAM32		–					
ADAM33		+	c-Kit ligand			Psoriasis susceptibility, asthma	[84]
ADAM34		–					
ADAMTS1	METH1	+	Versican, thrombospondin-1, semaphorin 3C, syndecan-4		Required for mammary tumor growth	Increased risk of bone metastasis in breast cancer	[85]
ADAMTS2	Procollagen N-proteinase	+	Collagen I and II N-propeptides			Skin fragility resulting from inactivating mutations	[86]
ADAMTS3	PCIINP	+	Collagen N-propeptides			Might encode for susceptibility to osteoarthritis	[87]
ADAMTS4	Aggrecanase-1	+	Versican			Expressed in human glioblastomas	[88]
ADAMTS5	Aggrecanase-2, ADAMTS11	+		Decreased susceptibility to induced arthritis		Tumor suppressor, antiangiogenic	[89]

ADAMTS6		–								
ADAMTS7		–								
ADAMTS8	METH2	+							Tumor suppressor, antiangiogenic	[90]
ADAMTS9		+		Died before gastrulation					Tumor suppressor	[91]
ADAMTS10		–								
ADAMTS12		+	Aggreccan, COMP					Increased angiogenesis and tumor cell invasion		[92]
ADAMTS13	vWF-cleaving protease	+	vWF	40–50% increase in vWF level					Acquired deficiency in Thrombotic thrombocytopenic purpura	[93]
ADAMTS14		+	Collagen N-propeptides						Increase in bone metastasis model of prostate cancer	[94]
ADAMTS15		+							Lower expression in breast cancer, inactivated in colorectal cancer	[95]
ADAMTS16		–								
ADAMTS17		–								
ADAMTS18	ADAMTS 21	–								
ADAMTS19		–								
ADAMTS20		–								

See text for the focused ADAM family members

Table 11.2 MMP family

	Common name	Main substrate	Phenotype and tumorigenesis in	Overexpression in human tumor	Reference
MMP-1	Collagenase-1	Collagen	Phenotype and tumorigenesis in knockout mice Decreased number and incidence of lung tumor in MMP-1a KO	Overexpression is associated with poor prognosis in colorectal, breast, and lung cancer	[98]
MMP-2	Gelatinase-1	Collagen IV, fibronectin, laminin	Reduced intraluminal leukocytes Decreased pancreatic carcinogenesis	Increased blood level in patients with breast or prostate cancer	[99]
MMP-3	Stromelysin-1	Basement membrane, E-cadherin, plasminogen	Decreased immune-complex-induced lung injury and neutrophil Mammary hyperplasia and cancer by overexpression	Expressed in the stromal compartment at all stages of lung tumor progression	[100]
MMP-7	Matrilysin	Fibronectin, plasminogen, proteoglycan, tenascin	Decreased transepithelial neutrophil migration in bleomycin-induced lung injury	Overexpression in pancreatic ductal adenocarcinoma	[101]
MMP-8	Collagenase-2	Collagen	Increased incidence of skin cancers	23 % of somatic mutations (reduced MMP enzyme activity are) found in melanoma patients	[102]
MMP-9	Gelatinase-2	Plasminogen, Collagen IV	Reduced alveolar bronchiolization after bleomycin Decreased skin carcinogenesis Decreased pancreatic carcinogenesis	Overexpression in prostate and lung cancer	[103]
MMP-10	Stromelysin-2	Collagen, fibronectin, laminin	Susceptibility to bacterial infection	Overexpression in lung cancer	[104]
MMP-11	Stromelysin-3	Fibronectin, laminin	Decreased mammary carcinogenesis, increased metastasis in MMTV-rasTG/MMP11 KO	Overexpression in breast cancer	[105]

MMP-12	Metalloelastase	Elastin, plasminogen	Reduction of matrix degradation by macrophage after subcutaneous implantation Increased lung metastases both in experimental and spontaneous model in mouse	Increased survival times in colorectal cancer patients	[106]
MMP-13	Collagenase-3	Collagen	Delay in endochondral ossification and vascularization of primary ossification centers Cartilaginous growth plate abnormality caused by accumulation of type X collagen Decreased subcutaneous tumor growth accompanied with reduced blood vessel density, and decreased incidence of lung metastases	Overexpression is associated with poor prognosis in colorectal cancer	[107]
MMP-14	MT1-MMP	Collagen, fibronectin, laminin, vitronectin, proMMP-2, proMMP-13, ADAM9, gelatin, fibrin/fibrinogen, perlecan, CD44, ICAM-1, tTG, LRP1, syndecan, av-integrin, C3b, EMMPRIN, ApoE, MICA, betaglycan, IL-8, SLP1, CTGF, DR6, DJ-1, galectin-1, Hsp90a, pentraxin, progranulin, Cys61, peptidyl-prolyl cis-trans isomerase A, dickkopf-1, Kiss-1, Dll1	Defects in skeletal development and angiogenesis Transgenic (MMTV-MMP14) mammary hyperplasia and cancer	High MMP-14 expression is associated with early death of breast cancer patients	[108]
MMP-15	MT2-MMP	Fibronectin, laminin, proMMP-2, perlecan, collagen, NC1, fibrin	Mice homozygous for a knock-out allele exhibit normal phenotype	Overexpression of MMP-15 in human lung adenocarcinoma compared with normal lung	[109]

(continued)

Table 11.2 (continued)

	Common name	Main substrate	Phenotype and tumorigenesis in	Overexpression in human tumor	Reference
MMP-16	MT3-MMP	Collagen, fibronectin, proMMP-2, gelatin, laminin-1, vitronectin, fibrin, α1PI, NgR1, Kiss-1	Phenotype and tumorigenesis in knockout mice	MT3-MMP is expressed in melanoma lymph node metastases	[110]
			Retard growth of the skeleton in the cranium and long bones	Overexpression of MT3-MMP in gliomas, hepatocellular carcinoma and gastric cancer	
MMP-17	MT4-MMP	Fibrinogen, fibrin, gelatin, pro-TNF, ADAMTS4, α2-macroglobulin, progelatinase-A	Abnormal diminished thirst	Breast cancer(+), MT4-MMP protein was particularly elevated in metastatic lymph nodes where both tumor and inflammatory cells displayed strong staining	[111]
MMP-19	RASI-1	Cartilage oligomeric matrix protein, aggrecan	Diet-induced obesity due to adipocyte hypertrophy	Increased expression during colorectal tumorigenesis	[112]
			Decreased susceptibility to skin tumor induced by chemical carcinogen. Increased tumor vasacularization		
MMP-20	Enamelysin	Amelogenin, collagen XVIII	Amelogenesis imperfecta	Overexpression in laryngeal squamous cell carcinoma	[113]
MMP-21	Homologue of xenopus XMMP	Unknown		Increased expression is associated with poor overall survival in gastric cancer patients	[114]
MMP-23	Cysteine array MMP	Unknown		Increased expression in melanoma is associated with poor response to immunotherapy	[115]
MMP-24	MT5-MMP	Unknown	Absence of mechanical allodynia after sciatic nerve injury	Overexpression in brain tumors	[116]
		proMMP-2, gelatin, fibronectin, laminin-1, CSPG, DSPG, KiSS-1, N-cadherin			

MMP-25	MT6-MMP	Fibrinogen, fibrin, fibronectin, collagen IV, gelatin, myelin basic protein isoforms, vimentin, cystatin C, collagen IV, procollagenase-A, chondroitin sulfate proteoglycan, dermatan sulfate proteoglycan, a1 proteinase inhibitor, urokinase plasminogen activator receptor	Elevated MT6-MMP mRNA is found in brain, colon, urothelial and prostate cancers	[117]
MMP-26	Endometase, matrilysin-2		In esophageal SCC, MMP-26 overexpression correlates strongly with depth of invasion, lymph node and distant metastases	[118]
MMP-27			In melanomas are frequently associated with mutations in Mmp8 and Mmp27 genes leading to loss-of-function and enhanced progression of the cancer95	[119]
MMP-28	Epilysin			[120]

See text for the focused MMP family members. Protease activity-independent functions are reported in MMP12, including antibacterial activity outside of the catalytic domain that disrupts bacterial membranes [121] and transcriptional activity for I κ B α by binding to its promoter after nuclear translocation to the nucleus [122]. I κ B α is engaged in export of IFN α . In both cases, MMP12 plays an antimicrobial role. Bioactive fragments derived from MMP-digested extracellular matrix are called matrilines, including VGV/APG, that cause monocyte migration and NF κ B activation [123, 124]. Therefore, MMPs are directly and indirectly involved in inflammation

Thus, both MMP and ADAM family proteins are critical mediators expressed not only in tumor cells but also in other stromal cells capable of changing the tissue microenvironment during tumor progression. In contrast to exogenous hemorrhagic snake venoms, they appear to be <endogenous blood lake makers> in Yoshida sarcoma.

I do not intend to explain all of the members of both families. I focus on some of them that are particularly important in tumor biology. The important point is that the potential substrates include molecules directly involved in ECM, cell adhesion, and immunity [125].

11.6.2.2 MMP (see Table 11.2)

MMP-1

MMP-1 degrades many types of collagen, such as type I as well as TGF β . Overexpression in lung cancer and colon cancer is associated with poor prognosis of the patients. Producers are tumor cells as well as stromal fibroblasts. Conversely, in a chemical carcinogenesis model of lung cancer with urethane, KO mice of MMP1a, the murine orthologous gene of human MMP1, turned out to be resistant in male but not female mice [98]. Flow cytometer analysis revealed that Th1 cytokines IL-27 and IFN γ , and IL-17 were up-regulated in the lungs of urethane-treated mice compared with those of wild-type mice. Precise linkage to up-regulation of Th1 cytokines and MMP1 is unknown, one thing is clear that the urethane-induced cancer development required MMP1 [98]. MMP-1 also is implicated in liver fibrosis [126].

MMP2

Overexpression is found in human breast cancer and prostate cancer. MMP2 cleaves laminin and various types of collagen, such as type IV, and is required for melanoma invasion. Not only melanoma cells but also DC express MMP2. MMP2 exerts its effect both protease activity-dependent and independent manners. MMP2, irrespective of its catalytic activity, binds integrin to induce OX40L expression, and active MMP2 cleaves IFNAR1 to block IFN β signaling for IL-12 expression. Therefore, MMP2 favors Th2 balance over Th1. Removal of N-terminal four amino acid residues of CXCL12 by MMP-2 resulted in loss of binding to its cognate receptor CXCR4 [127]. Furthermore, human MSC respond to G-CSF to secrete and activate MMP-2 [128].

MMP9

Overexpression is observed in lung cancer and prostate cancer. A variety of substrates are included, ECM such as type IV collagen, plasminogen, and ICAM-1 (Fig. 1.2 in Chap. 1). Apart from its function, a huge case-control study of 879 lung cancer patients in Spain revealed that MMP9-1562C/T polymorphism is significantly associated with protection against lung cancer development [129].

MMP-9 is a gelatinase expressed by leukocytes, which increases vascular permeability in steatotic liver ischemia and reperfusion injury [130]. MMP-9 gradually accumulated in bone marrow extracellular fluids when peripheral blood progenitor cells were mobilized following administration of G-CSF [131].

MMP13

In addition to MMP-2 and MMP-9, MMP-13 is up-regulated in hepatic fibrosis [132, 133]. MMP-13 plays a role in CXCL9-mediated liver fibrosis resolution [134].

11.6.2.3 ADAM (see Table 11.1)

ADAM10

In response to Ca^{2+} signaling induced by, for example, thrombin, ADAM10 is released from a calmodulin-bound form in the cytoplasm to become an active form in the membrane to exert its sheddase activity for the CD44 ectodomain engaged in cell adhesion to hyaluronic acid, thereby promoting cell migration and for VE-cadherin in endothelial cells facilitating vascular permeability [135]. A preferential ADAM10 inhibitor GI254023X blocked transmigration of PHA T-cell blasts expressing ADAM10 through HUVEC monolayer with VE-cadherin-mediated AJ.

In addition to CD44, ADAM10 cleaves the extracellular domain of another metastasis-related adhesion molecule L1-CAM whose expression and that of ADAM10 are up-regulated by β -catenin signaling in colon cancer cells [136]. HCT116 colon cancer cells stably expressing ADAM10 increased their metastatic ability when injected to spleen of nude mice.

Furthermore, ectodomain shedding has been reported in RAGE, receptor for AGE. Released soluble forms of RAGE can serve as a decoy receptor, modulating the RAGE-dependent biology [137]. In lungs in which RAGE is most abundantly expressed, both membrane-bound and cleaved forms of RAGE are detected in human samples.

ADAM12

Myofibroblasts express ADAM12 in the MMTV-PyMT tumor model and the W10 prostate cancer model. Mating with ADAM12-KO mice revealed requirement for ADAM12 in prostate cancer progression [138]. Critical substrates, such as HB-EGF, remains to be uncovered.

We have shown that ADAM12 binds, cleaves, and releases ephrin-A1 on endothelial cells into circulation. The soluble form of ephrin-A1 binds its own receptors EphA1 and EphA2 in lung endothelial cells to induce their co-endocytosis with VE-cadherin eventually leading to increased vascular permeability in the lungs, which facilitates the tumor cell entry into the lungs [139].

ADAM15

Colocalized with CD31 in lung endothelial cells, ADAM15 participates in the intensity of endothelial barrier function in a homeostatic manner [82]. Collagen type IV in the basement membrane is one substrate. LPS activates ADAM15 to weaken the barrier strength. Conversely, ADAM15-KO conferred resistance to LPS-induced vascular permeability [140].

ADAM17 (also called TACE)

Shedding of membrane-bound TNF α by ADAM17 (also called TNF α converting enzyme TACE) provide ligands with systemic bioavailability for TNFR, especially TNFR1. The TNF-TNFR system is complicated given that membrane-bound TNF α binds TNFR2 and ADAM17 cleaves both TNFR1 and TNFR2. Myeloid cell-specific conditional KO of ADAM17 rendered the mice resistant to LPS-induced septic shock [141]. ADAM17 activity is locked by constitutive binding to TIMP3, which is liberated by p38/Erk-mediated phosphorylation of ADAM17. TIMP3-KO mice are sensitive to LPS-induced septic shock [142].

ADAMTS1

Thrombospondin-1 (TSP-1) was undergone proteolysis by ADAMTS1 [143], and the cleavage products inhibited angiogenesis by sequestering VEGF and prevented VEGF from binding to the receptor [144]. It also has been reported that the inhibitory effect of TSP-1 on VEGFR2 is mediated by its 3TSR domain capable of binding to CD36. ADAMTS1 cleaves TSP-1 bound to ECM releasing the 3TSR domain-containing fragment. CT26 murine colon cancer cells secreting recombinant 3TSR showed diminished potentials to cause lung and liver metastasis when the cells were injected via tail and portal vein, respectively [145]. Conversely, *Adamts1*-deficient mice showed a decrease of mammary tumor growth and lung

metastasis in MMTV-PyMT mouse [146]. The inhibitory effect on metastasis in *Adamts1*-deficient mice may be mediated by the proteolysis of versican that shows promoting functions in metastasis [147, 148]. Taken together, ADAMTS1 has been shown both antiangiogenic and metastasis promoting effects.

ADAMTS2

The most investigated and characterized procollagen N-endopeptidase is ADAMTS2. It is mainly expressed in fibroblasts and cells of mesenchymal origin and its expression correlates with the expression of type I and type III collagens. Based on the ADAMTS2 knockout phenotype, suppression of TS2 may be good target for the fibrosis. The extent of fibrosis is reduced in TS2^{-/-} mice in comparison with their WT littermates. Inhibiting the maturation of fibrillar collagens may be a beneficial therapeutic approach to interfering with the development of fibrotic lesions.

ADAM metallopeptidase with thrombospondin type 1 motif 2 inactivation reduces the extent and stability of carbon tetrachloride-induced hepatic fibrosis in mice [149].

ADAMTS2 induces the apoptosis of endothelial cells by a mechanism independent of its catalytic activity but potentially related to interactions with a cell surface receptor. Furthermore, ADAMTS-2 strongly reduced branching of capillary-like structures formed by endothelial cells and their long-term maintenance and inhibited vessels formation in embryoid bodies (EB). ADAMTS-2 functions as an antiangiogenic and antitumoral molecule independently of its catalytic activity [150].

ADAMTS9: Potential Tumor Suppression

A genome-wide promoter methylation analysis revealed silencing of ADAMTS9 by methylation in many gastric cancer cell lines, including BGC823 and KatoIII. Ectopic expression of ADAMTS9 in BGC823 and subsequent transplantation to nude mice showed a tumor-suppressive ability of ADAMTS9. ADAMTS9 has no ability in proteolysis against TSP-1 and binding to VEGF. Accordingly, the inhibitory effect of ADAMTS9 on tumor growth may be different from that of ADAMTS1. Koo et al. concluded that ADAMTS9 contributed to capillary maturation and stabilization [151]. Critical substrates and precise mechanisms await elucidation.

ADAMTS13

Thrombotic thrombocytopenic purpura (TTP) has autoantibodies against ADAMTS13 that cleave vWF [152]. vWF is stored in Weibel-Palade body in

endothelial cells and α granules of platelets and is released as multimers. In order for vWF to function, the multimeric vWF needs to be cleaved by ADAMTS13. vWF protease activity was attenuated in patients with metastatic tumor, and multimeric vWF actually found in their plasma. Therefore, decreased proteolytic activity of ADAMTS13 against vWF may lead to a worse outcome [153].

11.6.2.4 MT-MMP

MT1-MMP (also called MMP14)

The following is a good example of an *ex vivo* model of cancer cell invasion. When tumor cells, such as MDA-MB-231 cells, were plated onto basement membranes (BM) collected from rat peritoneum, they perforate the BM by extending invadopodia of $\sim 0.8 \mu\text{m}$ into the subjacent interstitium [154]. An siRNA-mediated silencing of either MT1-MMP, MT2-MMP, or MT3-MMP, but not of MMP-2 or MMP-9, inhibited the invasion by up to 70%. Reexpression of MT1-MMP could rescue the inhibited phenotypes.

MT1-MMP activate MMP2 but not MMP9. Proteolytic activation of MMP2 by MT1-MMP is mediated by TIMP2 that binds both MT1-MMP and the hemopexin domain of MMP2 [155]. A peptide mimetic MMP inhibitor BB-94 and an endogenous MMP inhibitor TIMP-2, but not TIMP-1 capable of inhibiting secreted MMPs, that can block both MT1-MMP and secreted MMPs, such as MMP2, impaired the invasion. BM recovered from knockout mice of either MMP2 or MMP9 failed to affect the invasion excluding the possibility that BM-embedded MMP2 or MMP9 is actually responsible for the invasion.

Mice lacking TIMP1 (Timp1 $-/-$ mice) have provided additional evidence supporting the role of TIMP1 in control of ECM proteolysis within the cardiovascular system [156]. Although TIMP-2 is an inhibitor of MMPs, paradoxically in this mechanism it can function as a coactivator of proMMP-2 as well. Using TIMP-2-deficient mice and cells derived from them, TIMP-2 was shown to be required for efficient proMMP-2 activation both *in vivo* and *in vitro* [157]. TIMP4 expression is significantly lower in patients suffering from pelvic organ prolapse (POP), which supports a role for TIMP4 in protection of the ECM from increased proteolysis [158].

Both MT1-MMP and ADAM10 (see above) cleave the ectodomain of CD44 whose major receptor is hyaluronan, which induces cell migration. The binding between MT1-MMP and CD44s (standard CD44) is mediated by the hemopexin-like domain of MT1-MMP. This shedding is followed by another cleavage at the intra-membrane domain by presenilin-dependent γ -secretase. The released CD44 intracellular portion is translocated into the nucleus to activate transcription.

MT1-MMPs-dependent invasion through and degradation of BM and subjacent matrices not only generate bioactive peptide fragments but also allow interactions between interstitial ECM and integrin receptors on tumor cells.

MT1-MMP in Endothelial Cells (EC) [159, 160]

FGF-induced angiogenic response in cornea is defective in MT1-MMP-KO mice. Endothelial invadopodia, tip cells, is enriched with membrane-type matrix metalloproteinase MT1-MMP that not only degrades interstitial collagen directly but also activates MMP-2.

Substrate Specificity in MMPs

In the authentic *ex vivo* assay for sprouting and remodeling, the aortic ring model of rats and mice, genetic disruption of either MMP-2 or MMP-9 failed to affect the biological consequence. However, anti-MT1-MMP neutralizing antibody inhibited both sprouting and regression when added after sprouting. It is known that tissue inhibitors of MMPs (TIMPs) can suppress the function of MMPs. For example, TIMP-1 inhibits MMP-9, and TIMP-2 counteracts MT1-MMP. TIMP-2 but not TIMP-1 inhibited the angiogenic process in this model. Thus, it can be said that MT1-MMP working in concert with the functionally opposing TIMP-2 in a time-dependent fashion is critical in the whole process. Quantitative proteomics analysis of TNF α -activated endothelial cells derived from MT1-MMP-knockout mice revealed that one of the substrates of MT1-MMP is TSP-1.

RECK

RECK (reversion-inducing cysteine-rich protein with Kazal motif) is a membrane-anchored glycoprotein capable of directly inhibiting the enzymatic activity of MT1-MMP *in vitro*. RECK-KO mice die around E10.5 with vascular defect, which could be rescued by double KO with MMP-2 as judged by organization of collagen type IV and laminin in the endothelial basement membrane and of vascular structure. The human fibrosarcoma cell line HT1080 originally lacked RECK expression. When RECK was overexpressed in HT1080 and subcutaneously transplanted to nude mice, angiogenic sprouting was suppressed while the primary tumor size was not changed compared with that of nontransfected HT1080 [161].

MT1-MMP Is Shared by Endothelial Cells and Tumor Cells

In tumor angiogenesis, MT1-MMP plays a critical role in endothelial tip cells. Tumor cells also utilize MT1-MMP for local invasion. For example, a considerable population of MT1-MMP molecule is stored in the intracellular endosomes after internalization in MDA-MB-231 breast cancer cells. Focal delivery of MT1-MMP is accomplished by controlled exocytosis. The underlying mechanisms involve an interaction between the exocyst complex and the endosomal Arp2/3 activator

WASH (Wiskott-Aldrich syndrome protein <WASP> and Scar homolog) and actin-cortactin assembly [162].

New Strategies for Targeting MMP

The classical MMP inhibitors such as hydroxamic acid-based MMP inhibitors often have musculoskeletal syndrome (MSS) because of lacking specificity. MSS has been attributed to inhibition of MMP-1 and ADAM17 [163]. Recently, new strategies for MMP inhibitors with greater selectivity considering secondary binding sites (exosites) were developed. Aventis discovered a pyrimidine dicarboxamide that had a specific inhibitory activity for MMP-13 [164]. The Aventis compound binds within a specificity loop of the MMP-13 catalytic domain, which is recognized as an exosite [165]. Furthermore, Lauer-Fields et al. identified a novel, highly selective class of MMP-13 inhibitors, such as compound Q/4, Q1/20, and Q2/24, that have a more inhibitory potency for MMP-13 exosite than the Aventis compound and did not inhibit MMP-1 and ADAM17 [166].

EMMPRIN, an Upstream Regulator of MMP, in Glioma

EMMPRIN (extracellular matrix metalloproteinase inducer)/basigin/CD147 is a membrane-bound glycoprotein, which is mainly expressed in cancer cells and induces MMP from adjacent stromal fibroblasts. EMMPRIN expression was increased in glioma tissues compared with normal brain tissues, and its expression significantly correlated with the advanced stages of the disease [167]. To examine the involvement of EMMPRIN in the production of MMP-2, U251, a glioblastoma multiforme cell line, was co-cultured with brain-derived fibroblasts resulted in inducing not only MMP-2 but also MT1-MMP and MT2-MMP [168]. MT1-MMP can cleave membrane-bound EMMPRIN and release a 22 kDa soluble EMMPRIN fragment from tumor cells that had the MMP-2-inducing activity in fibroblasts [169]. The anti-sense-mediated knockdown of EMMPRIN has resulted in not only the down-regulation of MMP-2, MMP-9, and VEGF but also the defect of invasiveness in U251 glioblastoma cells [170].

MT2-MMP

MT2-MMP is expressed in a wide range of human cancer cells [171]. MT2-MMP cleaves fibronectin, laminin, proMMP-2, perlecan, collagen, NC1, fibrin, and nidogen [172–174]. MT2-MMP activates proMMP-2, which may promote basement membrane invasion [175].

MT4-MMP

MT4-MMP expressed by human breast cancer cells promotes the metastatic dissemination into lung by disturbing the tumor vessel integrity [176, 177].

MT6-MMP

Elevated MT6-MMP mRNA is found in brain, colon, urothelial, and prostate cancers [178]. MT6-MMP cleaves matrix metalloproteinases, gelatin, fibronectin, fibrin, and type-IV collagen [178–180]. A proteomic approach for neutrophil MT6-MMP shows a number of substrates for MT6-MMP, including the CC and CXC chemokines, fibrillin-1, extracellular matrix protein 1, syndecan-4, and type I, II, IV, and VI collagens, and galectin-1. MT6-MMP may regulate neutrophil and monocyte chemotaxis and by generating “eat-me” signals upon vimentin cleavage potentially increases phagocytic removal of neutrophils to resolve inflammation [181].

11.7 Vascular Mimicry and Angiotropism

Periodic acid (PA), a classical and reliable agent in pathology, reacts with carbohydrates, such as components in endothelial basement membrane, such as laminin and colors with Schiff’s (S) reagent. Red blood cells were found in the hollow of PAS-positive ECM in which no endothelial cells were recognized as judged by immunostaining of CD31, CD34, vWF, and VEGFR2. The matrix channels were formed by highly invasive, but not noninvasive, melanoma cells associated with neither endothelial cells nor fibroblasts and conducted dye in angiogram. cDNA microarray analysis between invasive versus noninvasive melanoma revealed up-regulation of Tie-1, uPA, and EphA2 in more than 70-fold in the invasive compared with noninvasive in the original report [182]. The proposed phenomenon vascular mimicry also was found in glioblastoma, in which PAS-stained channels were composed of SMA⁺CD31[−]VEGFR2⁺ tumor cells [183].

In 1997, transmission electron microscopy and immunohistochemical studies of human malignant melanoma revealed a direct contact of melanoma cells with endothelial cells from the abluminal side with laminin in between. This was called the angiotumoral complex [184]. The melanoma cells occupied the pericyte locations. Later with time-lapse video-microscopic analyses of murine tumors, it was shown that B16 melanoma cells or GL26 glioma cells migrate along the abluminal surface of the vessels and the angiotropism increased during the course of tumor progression.

Although it is not direct vascular mimicry, CD133⁺ glioblastoma stem cells often are found in perivascular niches and, surprisingly, shown to differentiate into pericytes [185]. TGFβ was shown to be necessary and sufficient for the

differentiation and SDF-1 stimulated the pericyte migration to endothelial cells in a pericyte CXCR4-dependent manner.

Generally, tumor vessels often lack pericytes and glioblastoma is well known for its hypervascularity. Anti-VEGFR2 antibody could normalize tumor vasculature increasing pericyte coverage by elevated expression of Ang1. Therefore, it was proposed that irradiation therapy should be performed during this vascular normalization window to avoid noxious effects by hypoxia due to tumor angiogenesis. However, in glioblastoma, selective killing of the tumor-derived pericytes was achieved by transduction of Desmin promoter-driven herpes simplex virus thymidine kinase (HSVTK) followed by ganciclovir (GCV) treatment, in which HSVTK converts GCV to a toxic metabolite to eliminate HSVTK-expressing cells.

EphA3 is highly expressed in glioblastoma stem cells and blocks their differentiation [186]. An shRNA-mediated EphA3 knockdown allowed neuronal and glial differentiation and instead inhibited tumorsphere formation.

NOS2, alternatively called iNOS, also is elevated in glioblastoma stem cells producing NO, which represses expression of a cell cycle inhibitor CDA1 [187]. A lipophilic NOS2 inhibitor BYK191023 that can penetrate the blood-brain barrier was shown to be effective for tumor growth in glioma xenograft model in nude mice.

11.8 Nerve and Tumor Cells

Tumor microenvironment is innervated, and from the nerve side perineural presence of tumor cells is a poor prognostic sign for many cancers, including head and neck tumors, pancreatic cancer, colorectal cancer, and prostatic cancer (see Fig. 9.4 in Chap. 9). To understand the interaction between nerves and tumor cells, we need to have a basic knowledge of anatomical structure of peripheral nervous system. Each axon, actual cord of neuron, is or is not wrapped by myelin sheath and Schwann cells and is separated by a connective tissue called endoneurium consisting of fibroblasts, mast cells, collagen and endoneurial fluid that correspond to cerebrospinal fluid in central nervous system. The whole bundle of those axons interspersed by endoneurium is surrounded by a group of flattened fibroblasts and collagen called perineurium. A collection of perineurium-surrounded bundles is further surrounded by epineurium in which blood vessels are found.

The original idea of perineural invasion (PNI) of tumor cells were proposed as tumor cells <in, around, and through> the nerves [188].

Here I discuss prostate tumors, for example. Quantification of nerve fiber densities as monitored by the expression of a neuron-specific marker neurofilament-L showing newly formed sprouting fibers, tyrosine hydroxylase (TH), that is responsible for synthesis of norepinephrine in sympathetic adrenergic fibers, and vesicular acetylcholine transporter (VACHT) indicating parasympathetic cholinergic fibers in 43 prostate cancer patient specimens showed that VACHT in intratumoral and TH in peritumoral tissues [189]. The intratumoral

nerve density had a positive correlation with the tumor proliferation index as judged by Ki-67 and extra-prostatic progression.

In a murine model of prostatic cancer in which PC-3 human prostate cancer cells stably expressing an imaging marker luciferase were injected into the ventral prostate of nude mice followed by analysis of tumor tissues after 11 weeks, neurofilament-L, TH, and VACHT were observed. Either functionally specific sympathectomy by 6-hydroxydopamine or physical one by surgical removal of hypogastric nerves selectively casting sympathetic stimuli abrogated tumor development. Under less tumor development, no distant metastasis was observed either. Given that tumor development was impaired in the genetic background of double deficiency in adrenergic receptor $\beta 2$ and $\beta 3$, adrenergic signaling of host-rather than tumor-origin appeared to play a significant role in tumor development. In the same model, a non-selective muscarine agonist carbachol enhanced and a nonselective antagonist scopolamine inhibited tumor invasion without affecting the cardiovascular hemodynamics. Given that no effect was found *in vitro* with same reagents, again the microenvironment is critical. When tumor transplantation was performed in nude mice crossed with those deficient in cholinergic muscarine receptor 1, lymph node invasion was compromised without affecting the tumor growth. Those findings were supported by the similar experiments in another murine prostate cancer model Hi-Myc transgenic mice in which c-Myc is overexpressed in their prostate under the control of the probasin promoter [190].

In both clinical and experimental settings, it is likely that stromal neuronal cells regulate development and invasion by sympathetic and parasympathetic stimuli, respectively. What is then the mechanism? It is not so simple. Primary tumor microenvironment is infiltrated by influential myeloid cells derived from bone marrow that also has a regulatory innervation to egress leukocytes after HSC differentiation, the tumor field extends to involve the primary site, bone marrow, peripheral nervous system, and perhaps even central nervous system. This reminds us of the old experiment in which lung metastasis of injected rat tumor cells through the tail vein was enhanced by an analgesic electrical stimulation of the midbrain in a manner independent of systemic administration of naloxone capable of blocking the analgesic effect [191].

To say the opposite, what do tumor cells do to nerve? Although perineural invasion could affect both motor and sensory function, I focus on the most noxious influence by cancer, pain. Intratumoral primary afferent nociceptors are stimulated by several ligand-receptor systems, including proton-TRPV1, endothelin-1 (ET-1)-ETAR, bradykinin (BK)-BKR1, NGF-NGFR, TNF α -TNFR, and Tryptase-PAR2. All of the ligands are directly secreted from tumor cells except for BK generated by kallikrein released from tumor cells and Tryptase secreted from mast cells.

For example, chronic application of NGF resulted in mechanical hyperalgesia in rat and transgenic mice that overexpress NGF in skin displayed mechanical hypersensitivity. What makes the situation more complicated is that NGF and NGFR are also expressed in tumor cells, such as pancreatic cancer supporting not only pain but also their enhanced proliferation with perineural invasion [192].

11.9 Fibroblasts and Tumor Cells

Fibroblasts are spindle-shaped and express fibroblast-specific protein1 (FSP1) that is associated with cytoskeleton. Forced FSP1 expression in epithelial cells converted their morphology to that of fibroblasts [193]. Collagenase- and hyaluronidase-mediated dissociation followed by differential sedimentation of resected human breast cancer samples provided carcinoma-associated fibroblasts (CAFs) for experimental use [194, 195]. When transplanted in mixture with human breast cancer cell line MCF-7 ras in xenograft model, they enhanced tumor growth compared with fibroblasts from normal breast tissues. The molecular mechanisms entail expression of CXCL12 in CAF and its receptor CXCR4 in both cancer cells and bone marrow-derived endothelial progenitor cells that eventually promote tumor angiogenesis as demonstrated by inhibition of tumor growth by lentivirus-mediated gene transfer of anti-CXCR4 siRNAs.

An intriguing but still contentious idea is that genetic mutations can take place in both fibroblasts and endothelial cells in primary tumor microenvironment and contribute to tumor cell proliferation [196, 197]. Given the hypoxic, acidic, and ROS-abundant conditions, stromal cells also may acquire genetic alterations to either get apoptotic or actively secrete growth-promoting molecules even without endless mitosis of their own. Alternatively, growth factors produced by tumor cells, including FGF and VEGF, continuously stimulate or force proliferation of stromal cells to achieve senescence eventually. SASP is represented by production of IL-6 and IL-8.

The origin of CAF is under debate and may come from differentiated MSC, pericytes that share many markers with CAF, including SMA and NG2, or tissue resident fibroblasts transformed by the adjacent tumor cells.

CAF under aerobic glycolysis and glutaminolysis secrete lactate and NH₃, respectively (see Chap. 10). Lactate inhibits both PHD2 and I κ B, which results in released activation of HIF-1 α and NF κ B, respectively [198]. HIF-1 α - and NF κ B-driven expression of VEGF and IL-8, respectively, eventually support tumor microenvironment. Endothelial cells that are stimulated by IL-8 trigger VEGF-VEGFR2 autocrine activation.

11.10 Quiescence of Cells in Niche

I describe molecular basis of a special microenvironment called niche in which stem cells reside in quiescence, at the G0 phase of cell cycle (see Chap. 10, senescence vs quiescence). Every tissue contains its own stem cells from which all type of cells that participate in the formation of the tissue architecture are supposed to stem. Tumor is not an exception given the assumption of cancer stem cells (CSC) (see below). Recent accumulation of evidence on hematopoietic stem cells (HSC) and their niche in bone marrow provides a good reference to think

about niche for CSC [199]. CSC niches play an important role not only in maintenance of stem cell properties, such as quiescence, but also in evasion from immune surveillance and metastasis and consist of stromal cells, such as cancer-associated fibroblasts (CAF), MSC, tumor-associated macrophages (TAM) or neutrophils (TAN), MDSC, vascular system, and ECM. HSC and EC provide us with good lessons.

11.10.1 HSC

11.10.1.1 Niche Cells

The idea of stem cell <niche> was initially proposed by R. Schofield in 1978 as <a fixed tissue cell> that is in association with and prevents maturation of HSC in bone marrow [200]. The current idea is that HSC niche is composed of several types of cells and ECM, including endosteal osteoblasts and their progenitors, nestin⁺ mesenchymal stem cells (MSC), sinusoidal endothelial cells, macrophages, and sympathetic neurons. The roles played by those elements are to regulate the switch between quiescence, self-renewal, and differentiation. Homeostatic behaviors or maintenance of HSC, including long-term repopulating activity, appropriate localization and number of HSC in bone marrow, disruption of quiescence, and self-renewal has been evaluated in mice deficient in a number of genes and many necessary conditions have been established. Those conditions are at the level of external stimuli to HSC, gene expression in HSC, and molecules involved in posttranslational regulation (summarized in Fig. 11.3).

For example, Angiopoietin1 (Ang1) stimulation of its tyrosine kinase receptor Tie2 expressed in Tie2⁺ LSK cells represses expression of CXCR4, VEGFR2, etc., and enhances β 1 integrin and N-cadherin that gives adhesion to bone-lining osteoblasts [203]. The niche in which osteoblasts are engaged is called <osteoblastic niche.> This takes place in a tyrosine kinase activity-dependent manner and mice doubly deficient in Tie2 and its homology Tie1 display impaired maintenance of HSC in bone marrow.

The second binding interaction shown to be required for HSC maintenance is that between thrombopoietin (TPO) produced from osteoblastic cells and its receptor MPL expressed in MPL⁺LSK cells [204]. The stimulation induces increased expression of TPO, Tie2, and the CDK inhibitor p57. Conversely, in TPO-KO mice, the HSC number was diminished by 150-fold. Of note, osteoblastic niche is dispensable given that hematopoiesis can be achieved in extramedullary sites as found in enlarged spleen in CML and rarely in lungs after allogeneic stem cell transplantation.

Whatever the external stimuli are, HSC quiescence absolutely requires cell cycle arrest at G0 in which the CDK4/6 inhibitors p27 and p57 play an essential role [205]. During the course of LSK cell differentiation from LT-HSC (CD34⁻Flt3⁻Tie2⁺ or CD150⁺CD48⁻), ST-HSC (CD34⁺Flt3⁻ or CD150⁻CD48⁻), and

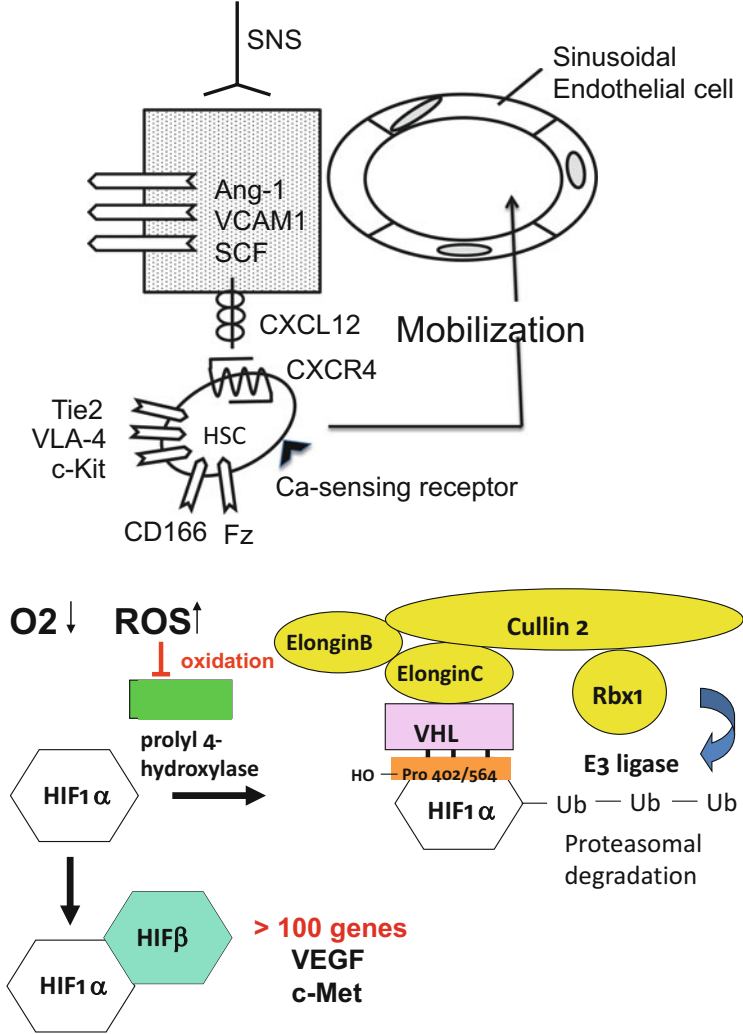


Fig. 11.3 HSC soil and hypoxic response. CD166, a member of Ig superfamily, by homophilic interaction, also is engaged [201]. CD166-KO LSK engrafted poorly wild-type mice and CD166-KO host failed to support LT-HSC. Noncanonical Wnt pathway including Flamingo and Frizzled (*Fz*) is further involved (see text). HSC retention protein CXCL12 is supported by CD169⁺ macrophages and HSC egressed in CD169-KO mice [202]

to MPP (CD34⁺Flt3⁺CD48⁺) where Flt3 is also called Flk2 and CD150 and CD48 belong to the SLAM family receptor [206], expression levels of p57 go down. When LT-HSC is stimulated with SCF, p57 is degraded and instead the CDK activator D1 cyclin enters into the nucleus to drive cell cycle. The import of both D1 and p57 into the nucleus is chaperoned by Hsc70 in a manner dependent on what we have

proposed as the nuclear localization related signal (NLRS) of Hsc70 [207]. In doubly knockout of p27 and p57, HSC quiescence is abolished.

Irving L. Weissman group showed in 2003 that canonical Wnt signaling could expand the pool of HSC by experiments in which constitutively activated β -catenin was retrovirally transduced in sorted Thy-1.1^{low} LSK cells to up-regulate expression of HoxB4 and Notch1 (see below) [208]. Conversely, β -catenin-KO bone marrow displayed no change in cellularity but decreased number of osteoblasts and VCAM-1 expression, and decreased ability to maintain LSK cells in long-term engraftment of 16 weeks in congenic transplantation experiments with CD45.1 and CD45.2 strains (see Chap. 12) [209]. In homeostatic conditions, N-cadherin⁺ osteoblasts predominantly express non-canonical Wnt ligands, such as Wnt11 and Wnt16, but not canonical ones like Wnt3a, which inhibit the NFAT-IFN γ pathway through both Frizzled 8 (Fz8) and Flamingo (Fmi) receptors. LT-HSC is decreased in Fmi-KO. Under stress, such as 5-FU, expression of the noncanonical Wnt ligands is suppressed concomitant with increased expression of canonical ligands, such as Wnt7a, and of inhibitors like Wif1 against the noncanonical ligands, resulting in deinhibition of the NFAT pathway and β -catenin activation in favor of cell proliferation as monitored by reduced expression of p57 and PTEN [210].

Most LT-HSC is associated with sinusoidal endothelial cells that express CXCL12 (alternatively called SDF-1) (CXCL12-abundant reticular cells, CAR cells), which stimulates its receptor CXCR4 on HSC [211]. Disruption of the CXCL12-CXCR4 system resulted in mobilization of HSC and progenitor cells from bone marrow. Conditional knockout of CXCL12 from candidate cells in niches was performed in endothelial cells (mediated by Tie2-Cre transgene), mature osteoblasts (osteocalcin-Cre), osteoprogenitors and CAR cells (osterix-Cre), and multipotent mesenchymal progenitor cells (Prx1-Cre) [212]. It turned out that deletion of CXCL12 in nestin-negative mesenchymal progenitors resulted in loss of HSC, its quiescence and long-term repopulating ability.

Adhesion molecules engaged in this vascular niche lodgment of HSC are VLA-4 on CD150⁺CD244⁻CD48⁻ HSC and VCAM-1 on sinusoidal EC.

Study on signals that confer abilities on LT-HSC to self-renew and serially repopulate has been hampered by the difficulty to co-culture endothelial cells in vitro with HSC without affecting HSC by factors that support endothelial survival. For example, if you put bFGF in the culture, potentials of endothelial to control HSC can be hidden by the effect of bFGF on HSC. That was overcome by the establishment of human primary endothelial cells expressing the adenoviral E4ORF1 gene [213]. Those cells could be co-cultured with murine HSC free of both serum and cytokines but only with sKitL. sKitL induces HSC to secrete VEGF, which stimulates expression of Notch ligands, such as Jagged-2 in endothelial cells in a VEGFR2- and VE-cadherin-dependent manner. The Notch ligands then exerted Notch signaling in HSC in a direct cell-cell contact-dependent fashion. HSC doubly deficient in Notch1 and Notch2 failed to do so and therefore was exhausted [214].

Briefly, both Notch family of proteins and their ligands are transmembrane proteins. Notch undergoes cleavages upon cell-cell contact by TACE (also called ADAM17) and presenilin in the γ secretase complex, allowing Notch intracellular

domain (NICD) translocation to the nucleus without any help of signaling molecules where it functions as a transcription factor to induce expression of a variety of genes, including p21, a cell-cycle inhibitor presumably responsible for quiescence, and Myc. In the primary keratinocyte system, Notch1 is required for p21 induction [215].

Bone marrow is thought to be a naturally hypoxic tissue while tumors are pathologically hypoxic as described later. There are a number of techniques by which to detect hypoxia *in vivo* [216]. Anti-HIF immune staining and incorporation of pimonidazole that binds protein adducts in hypoxic conditions are useful for molecular biologists. The assumed critical difference between osteoblastic and vascular niches is that oxygen tension is lower in osteoblastic than vascular niche closer to circulation. The actual oxygen tension in bone marrow of normal volunteers is 7.2% as immediately assessed by bone marrow aspirates although the results lacked information on spatial distribution of oxygen [217]. The physiological level of pO_2 is assumed to be even lower in osteoblastic niches as suggested by favorable *ex vivo* cultures of HSC in 1–3% oxygen tension. Recently, two-photon phosphorescence lifetime microscopy enabled direct *in vivo* measurements of local oxygen tension in murine bone marrow [218]. Vessels penetrate from bone to bone marrow cavity. A mean value was 2.7% (1.5–4.2%) at intravascular sites. The values were 1.3% in perisinusoidal regions $>40\ \mu\text{m}$ from the bone, and 1.8% outside the vessels in the endosteal regions. Interestingly, transplanted LKS cells did not necessarily home to the low oxygen concentration sites.

LT-HSC is positive in hypoxic cell marker pimonidazole *in vivo*. Proline hydroxylase activity of PHD2 is highly dependent on oxygen. Its substrate HIF-1 α is hydroxylated this way in normoxic condition, which allows HIF-1 α to bind the ubiquitin E3 ligase complex VHL and subsequently get ubiquitinated for proteasomal degradation (Fig. 11.3). In hypoxia, de-inhibition of HIF-1 α activates transcription of 100–200 genes. For example, the ABC transporter BCRP-1 is expressed in HSC, which provides an experimentally useful HSC marker when tested with Hoechst dye-efflux. The essential role of regulated expression levels of HIF-1 α in the maintenance of quiescence has been shown by the experiments using VHL-KO and conditional KO of HIF-1 α [219].

As expected mitochondrial oxygen consumption in HSC is reduced to approximately 10% of other cells. As judged by CD34 $^+$ human cord blood HSC, mitochondrial mass-high populations are more quiescent and more self-renewing and more repopulating in immune-deficient mice.

HSC need to be collected before transplantation. Collection in ambient oxygen gives poor recovery of HSC with long-term repopulating ability and manipulating bone marrow under hypoxia is proposed [220]. The oxygen shock induced the mitochondrial permeability transition pore (mPTP) to generate ROS thereby damaging HSC exposed to air in a manner dependent on cyclophilin D (see Chap. 10). Cyclosporin A can rescue the deficit by binding cyclophilin D and blocking the opening of mPTP.

Here are examples of HSC mobilization by immunological dangers.

It is known that LPS can induce splenomegaly. TLR4 is expressed in HSC and the reconstitution ability was reduced in HSC derived from TLR4-KO mice [221]. The extramedullary hematopoiesis in spleen was explained by the experimental results that GFP-labeled LSK cells with expression of both VLA-4 and TLR4 were in a close contact with VCAM-1-expressing resident macrophages in the splenic red pulp in LPS-primed mice. LSK cells were mobilized and kept in the splenic niches by TLR4 and VLA-4, respectively. Given that spleen is highly vascular and that growing GFP-LSK cell colonies were found, splenic macrophage-based niches for HSC allow proliferation and differentiation of HSC [222].

In an excellent review by Andreas Trumpp's Lab in 2011, it is assumed that CAR cells may have nestin⁺ MSC as subpopulation [223]. MSC can self-renew in serial transplantation, multipotent to spontaneously differentiate into mesenchymal lineage of cells. It was reported that 60% of LT-HSC is directly adjacent to nestin⁺ MSC (MSC niche) [224]. Nestin⁺ MSC, as judged by GFP⁺ cells whose expression is regulated by the nestin promoter, is perivascular stromal cells constituting roughly 4% of stromal CD45⁻ cells and 0.04–0.08% of nucleated cells in bone marrow, lacks expression of CD31 (= PECAM), CD34, and VE-cadherin. Both TLR2 and TLR4 are found in MSC, and their authentic ligands Pam3Cys and LPS could induce IL-6 secretion through NFκB activation [225]. Pam3Cys, but not IL-6, inhibits spontaneous adipogenesis from MSC. MSC cross-talks not only with macrophages but also sympathetic neurons.

Depletion of CD169⁺ macrophages induced HSC egress from bone marrow, suggesting that this type of macrophages is necessary for retention of HSC in bone marrow [21]. On the other hand, sympathetic nervous system stimulates HSC egress.

11.10.1.2 Neuron

Microenvironment of HSC in bone marrow is innervated by sympathetic nervous system and β3-adrenergic receptor-mediated circadian oscillation of CXCL12 expression is observed [226]. Pharmacological disruption of noradrenergic neurons by 6-hydroxydopamine (6OHDA) or the genetic background of knockout in dopamine β-hydroxylase (DBH), which is responsible for conversion of dopamine to norepinephrine, significantly impaired G-CSF-induced egress of HSC and progenitor cells [227]. The mobilization defect in DBH-KO mice was restored by β2 agonist clenbuterol.

What is nestin? Nestin is intermediate filaments that participate in eukaryotic cytoskeleton whose expression is developmentally regulated and serves as a marker of neuronal stem cells (NSC). Targeted disruption of the nestin gene causes embryonic lethality with 90 % penetrance. Nestin-KO cells isolated from E11.5 embryos show reduced self-renewal and are prone to apoptosis, which cannot be blocked by pan-caspase inhibitor z-VAD [228]. Vimentin-KO mice display no overt phenotype. Given that nestin cannot polymerize into intermediate filaments

in vimentin-KO conditions, and that vimentin-KO cells fail to acquire apoptotic phenotypes, cytoskeleton-independent functions of nestin are assumed. Nestin⁺ cells are frequently found in regenerative tissues.

Molecules involved in gene expression such as transcription factors and microRNAs also play regulatory role in HSC maintenance. For example, Myc proto-oncogene is necessary and sufficient for HSC maintenance. The HSC number in bone marrow is high in conditional Myc-KO and low with impaired self-renewing and active differentiation in Myc overexpression [229]. This contrasts with KO of tumor suppressor p53 in which HSC reentry into cell cycle is observed in a manner independent of p53 target gene p21. Loss of other transcription factors such as HIF-1 α and ERG also results in HSC exhaustion due to reentry into cell cycle [230].

In addition, HSC express calcium-sensing receptor (CaR) and CaR-deficient cells fail to be localized in endosteal niche when transplanted into normal mice [231].

HSC need to defend against ROS for their functional maintenance by utilizing one of the DNA repair systems: NHEJ (see Chap. 10). While RAG-KO mice in which V(D)J but not NHEJ is defective show normal HSC and normal niche, SCID mice in which DNA-PK and therefore NHEJ is impaired display defective HSC but normal niche (see Chaps. 9 and 10).

11.10.2 Endothelial Cells

Endothelial cells in physiological conditions also are quiescent for years [232]. Their high plasticity is evident when the long-term quiescence is switched to active proliferation by VEGF for example. Given that angiogenesis is the formation of new vessels from the preexisting ones, VEGF-stimulated growing and migrating cells at the tip of angiogenesis (tip cells), the phalanx endothelial cells that keep quiescence and in-between stalk cells need to co-exist. VEGFR2 stimulation in tip cells results in increased glycolysis by PFKFB3 that produces fructose 2,6 biphosphate, a potent allosteric activator of PFK1, and Akt-dependent biogenesis in mitochondria (see Fig. 10.3 in Chap. 10). However, at the same time those tip cells up-regulate Notch ligand Delta-like 4 (DLL4) in stalk cells, which down-regulates VEGFR2 and PFKFB3-dependent enhancement of glycolysis [233]. In quiescent endothelial cells, glycolysis is decreased with low oxidative metabolism. This is assumed to be one of the protective mechanisms against high oxygen concentration in circulation that may cause detrimental effects by ROS on those cells.

Although sufficient elucidation of molecules essential for maintenance of endothelial quiescence needs further study, antagonistic factors against VEGF and other angiogenic factors, such as FGF, may provide us with a clue. The following are two examples.

Sprouty 2, an antagonistic adaptor protein against FGF whose precise functions remain to be uncovered, is up-regulated in mouse aortic endothelial cells when they undergo contact inhibition while down-regulated to hardly detectable levels in single cell conditions [234]. Extracellular Ca^{2+} depletion disrupts N- and VE-cadherin-mediated adhesion of adherens junctions, which destabilizes Sprouty 2. Vascular permeability factors other than VEGF and FGF, $\text{TNF}\alpha$, and thrombin, for example, down-regulate Sprouty 2. In Sprouty 2-KO cells, FGFR-Erk2 signaling, which in turn activates the VEGF-VEGFR2 cascade, is sustained to induce over-proliferation without contact inhibition. It can safely be said that Sprout 2 is required for maintenance of confluence based on endothelial quiescence *in vitro*.

Nuclear IL-33 (alternatively called ILF11) in endothelial cells disappears upon acute injury or inflammation in which endothelial cells need to proliferate, and remains absent until they reach contact inhibition by p27 [235]. Once released from damaged cells into extracellular space IL-33 serves as an alarmin. One of the Notch ligands delta-like protein 4 (Dll4) is generally expressed in all segments of the human vasculature and Notch 1 signaling induces IL-33 expression. This is consistent with the finding that angiopoietin-1 induces endothelial quiescence by promoting Dll4-mediated signaling.

Another molecule that promotes endothelial cell quiescence is R-Ras that is highly expressed in mature and quiescent endothelial cells and conversely down-regulated in tumor vessels [236]. Readers should remember the involvement of Rho, another small GTPase, in vascular permeability (see Chap. 3). When B16F10 melanoma cells were implanted in mice, the tumor displayed increased vascular permeability and deposition of fibrinogen and fibrin owing to severely impaired collagen IV in the basement membrane. Add-back experiments showed that R-Ras expression restored the endothelial barrier function back to those vessels by suppressing the internalization of VE-cadherin. R-Ras expression in pericytes also facilitated the interaction between those and endothelial cells to reinforce the vascular barrier.

CD105 (endoglin) is an auxiliary receptor for $\text{TGF}\beta$. CD105 is up-regulated in endothelial cells during leukocyte extravasation and binds leukocyte $\alpha 5\beta 1$ integrin through its RGD sequence, thereby promoting transendothelial migration [237]. LPS-stimulated leukocyte migration to lungs was significantly reduced in CD105^{+/-} mice compared with wild-type (CD105-KO mice die at E10.5–11.5). The inhibitory effect of $\text{TGF}\beta$ on endothelial cells requires CD105. In endothelial cells of CD105^{+/-} mice, reduced expression of VE-cadherin and enhanced proliferation also were observed [238]. Mutations were reported in Osler-Weber-Rendu disease (also called hereditary hemorrhagic telangiectasia type1 <HHT1> syndrome) with vascular dysplasia [239].

Endothelial cells that shift between quiescence and proliferation can also reach senescence especially by the effect of ROS produced from mitochondrial complex I and III. To maintain the endothelial homeostasis, excessive ROS generation is balanced by an inhibitory activity of prohibitin-1 on the complex I, which eventually prolongs the time before senescence [240]. What would happen when endothelial cells reach senescence in for example tumor microenvironment after forced

proliferation by abundantly secreted VEGF from tumor cells and macrophages? Here is an experiment. When HUVEC becomes senescent they enlarge by stop growing even in the presence of acidic FGF. From the time of primary isolation of HUVEC, the total number of times the population doubles in vitro (population doubling) was more than twofold when expression of IL-1 α was down-regulated by antisense technique [241].

In normal human fibroblast HCA2 from neonatal foreskin, it was shown that binding of membrane-bound IL-1 α , which is highly expressed in senescent cells, to IL-1R induced expression of IL-6 and IL-8, which could be blocked by either IL-1ra or anti-IL-1 α antibody. In double strand DNA damage-induced senescence by bleomycin, robust expression of IL-6 and IL-8 was observed in 3–4 days, which was followed by IL-1 α - but not IL-1 β -dependent up-regulation of miR-146a in 12–25 days suppressing IRAK-1 expression [242]. This is supposed to be a negative feedback for the inflammation by SASP (see Chap. 10 and Chap. 14).

What would happen when endothelial cells were injured in a sterile fashion? Irradiation-induced DNA damages can put cells into cell death or senescence, and activates both transcription factors p53 and ATF3, both of which induces up-regulation of plasminogen activator inhibitor-1 (PAI-1) although the precise switching mechanisms are still unknown [243, 244]. Irradiation at 18 Gy of intestinal segments in PAI-1-KO mice displayed resistance against cell death, which was accompanied by elevation of anti-apoptotic BCL-1 and BCL-XL. Conversely, HUVEC overexpressing PAI-1 was sensitive to irradiation at 10–20 Gy in vitro. In addition to its anticoagulation roles by inhibiting t-PA and u-PA (see Chap. 3), PAI-1 up-regulation under stress such as endothelial injury may either put endothelial cells into senescence in the case of less and fixable DNA damages or into apoptosis when unfixable. The role of PAI-1 in induction of replicative senescence in mouse and human fibroblasts also is known.

11.10.3 Aberrant Stem-Cell Pathways in Tumor Cells

11.10.3.1 CSC: Definition and Overview

As proposed, CSC define a subpopulation of tumor cells within a tumor that has the ability to self-renew and to regenerate tumors in immune-deficient mice. In addition to the criteria, CSC have interesting features including high efficiencies in metastasis (see below). Although CSC just seemed to produce proliferating progenitors by analogy to HSC, current understanding is that there is an interconversion between CSC and non-CSC in tumors. As stated earlier, CML is a stem cell disease (see Chap. 9). CML-GMP derived from CML-HSC can self-renew. In tumors, namely nonleukemic cancers, it is difficult to demonstrate their epithelial stem cell origin of the corresponding organ. Comparison of efficiency in MMTV infection-mediated tumorigenesis of mammary epithelial cells between wild-type mice and those with WAP-TGF β 1 transgene (tg) that caused premature stem cell

senescence revealed that TGF β 1 tg mice had resistance to mammary tumor development, suggesting that malignant transformation of cells may take place at stem cell levels [245]. Plasticity from non-CSC to CSC also was shown in human basal breast cancer cells (see Chap. 9) injected into the mammary fat pads of NOD/SCID mice, which was dependent on ZEB1 as judged by CD44^{low} to CD44^{high} conversion [246].

Here I discuss relevance of HSC niche to cancer. We can easily recognize analogy between mobilization of leukocytes, whether HSC or their progenitors, from bone marrow and intravasation of tumor cells, whether cancer stem cells or their progenitors after EMT, into circulation. Lessons from HSC that we should learn were nicely reviewed, including the issue of hypoxic response by HIF1 and metabolic program, such as LKB1 [247, 248].

Hockel and Vaupel found that the median oxygen tension ranges from 10 to 30 mmHg in tumors and proposed tumor hypoxia as defined by the tissue oxygen tension below 10 mmHg (1.3%) [249]. In bone marrow samples from CML patients HIF-1 α expression is stronger in blast crisis than chronic phase, and imatinib only partially diminished HIF-1 α activation of target genes such as VEGF-A and GLUT1 in a posttranscriptional manner when tested with CML progenitor cells in vitro [250]. Although low oxygen tension and high HIF-1 α expression were observed in bone marrow samples from multiple myeloma (MM) patients their levels were similar to those from healthy volunteers [251]. However, MM model animals such as 5T33MM mice revealed more prominent hypoxia than wild-type controls as judged by markers of HIF-1 α and pimonidazole [252]. The bone niche permissive for myeloma cell propagation contains osteoclasts, activity of which is up-regulated by myeloma cells and can be reduced by zoledronic acid or antibody against RANKL expressed in myeloma cells [253].

During the propagation of tumor angiogenesis, hypoxia inevitably takes place at least in certain sites within the tumor tissue at certain time points (see Fig. 10.3 in Chap. 10). In this pathologically hypoxic tumor tissue, execution of TGF β and Wnt signaling contributes to the activation of genes responsible for stemness of the tumor cells. Whereas hypoxia-induced ROS (see Chap. 10) activates NF κ B signaling, HIF-1 α suppresses Myc activation that stimulates cell cycle (see complicated linkage between Myc and HIF-1 α in Chap. 10). Therefore, appearance and disappearance of hypoxic sites may regulate inter-conversion between cells with and without stem cell features and cancer stem cell niche formation [201].

Because systemic effects towards and from the whole body in primary tumor are discussed from the standpoint of inflammation in Chap. 12, I herein focus on significance of essential HSC niche-organizing molecules in cells in the primary microenvironment but not on mobilization by itself.

I introduce just some of the informative experiments to get insights into the relatedness between them.

11.10.3.2 Tie2 and MPL

Contribution of Tie2 and MPL in tumor progression is not clear. Analysis of 38 canine adrenocortical tumors by qPCR and immune-staining for angiogenesis-related gene expression revealed significant elevation of angiopoietin 2 but not VEGF and Tie2 [254]. A report shows that MPL expression levels were under detection in 105 of the 118 advanced breast cancer samples and were very low in 48% of the 29 non-small cell lung cancer samples [255].

11.10.3.3 CaR

Analysis of 33 patients with metastatic progression to bone ($n = 11$) or lungs ($n = 11$) or without ($n = 11$), Ca-sensing receptor expression levels were 7.9-fold and 3.7-fold higher as judged by qPCR and flow cytometry, respectively [256].

11.10.3.4 CD117 (c-Kit)

Evaluation of abilities for self-renewal and metastasis in CD117⁺Stro-1⁺ cells vs CD117⁻Stro-1⁻ cells derived from mouse and human osteosarcoma cell lines, including K7M2 cells, where Stro-1 is known to express exclusively in MSC, revealed that the CD117⁺ population was enriched in CXCR4 expression and more metastatic to lungs and liver on orthotopic injection in NOD-SCID mice [257].

11.10.3.5 VLA-4 ($\alpha 4\beta 1$ Integrin)

VLA-4 also serves as a receptor for fibronectin. In 1988, coinjection of fibronectin peptide RGDS with B16F10 melanoma cells into the tail vein reduced lung metastasis [258]. Although their experimental system did not analyze any primary tumors and they observed no change in the efficacy of antimetastasis in thrombocytopenia or beige, i.e., NK cell-deficient background, rather fast loss of radiolabeled melanoma cell retention in the lungs and short plasma half-life of the peptide of 8 min strongly suggests VLA-4-mediated tumor cells homing in the pulmonary structures, such as vasculature. VCAM-1, one of the ligands of VLA-4 (Figs. 11.3 and 11.4; and Fig. 1.2 in Chap. 1), belongs to the NF κ B-regulated genes. VLA-4 on CML cells (see Chap. 9) binds VCAM-1 on bone marrow stromal cells, which results in NF κ B activation to up-regulate expression of IL-6, VEGF and PlGF [259]. The biological role of VLA-4 is also played in pre-metastatic microenvironment (see Chap. 13).

Migrating cells	Receptor — Ligand	Niche cells
HSC	VLA4 — VCAM-1	Osteoblast EC macrophage(spleen)
CML-HSC	VLA4 — VCAM-1 VCAM-1	CML macrophage non-CML EC
BMDC	VLA4 — VCAM-1 Fibronectin	EC
tumor cells	VLA4 — VCAM-1 VCAM-1 — VLA4	EC BMDC

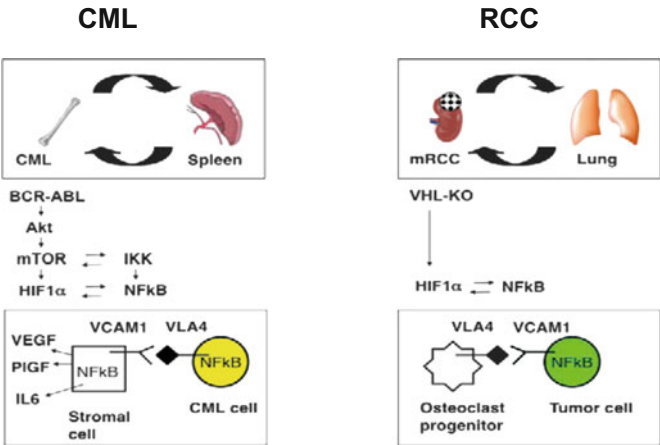


Fig. 11.4 VLA4-VCAM-1 system. VLA-4 is an example of factors that affect niche formation in both HSC and tumor/leukemic cells. The VLA4-VCAM1 adhesion system is commonly used in HSC, BMDC (bone marrow-derived cells), and tumor cells. In metastatic renal cell carcinoma (RCC), loss of function of VHL (see Fig. 11.3) activates HIF-1 signaling even in normoxic conditions. In both cases, the VLA4–VCAM1 interaction has antiapoptotic effects. EC (endothelial cells)

Pseudo-hypoxia and Pseudo-starvation

I underline the increasing significance of the close connection between inflammation and metabolism. HIF-1α is stabilized in normoxia when prolyl hydroxylase (PHD) is inhibited by succinate or when VHL is genetically defective as I stated in Chap. 10. The pseudo-hypoxic circumstance also can take place when HIF-1α is activated by NFκB signaling. Macrophages stimulated by LPS showed transcriptional activation of the HIF-1α gene in an IKKβ-dependent manner [260]. While VHL loss-mediated EMT (epithelial to mesenchymal transition) (see below) in renal carcinoma cells took place dependently on NFκB, introduction of a VHL-resistant HIF-1α mutant

(continued)

augmented NF κ B activity with manifestation of EMT [261]. Conversely, hypoxia induced NF κ B up-regulation in a HIF-1 α -dependent manner in neutrophils [262]. In tumor microenvironment, reciprocal activation between HIF-1 α and NF κ B appears to exist. If true, both tumor cells and stromal cells as exemplified by macrophages in aerobic microenvironments are supposed to be under pseudo-hypoxia. Activation of the glycolytic pathways in tumor cells even in aerobic conditions changes the ATP/AMP ratio activating AMPK (see Chap. 10) causing pseudo-starvation [263].

11.10.3.6 Nestin

Nestin + oligodendroglioma and glioblastoma cells were found to be closely associated with endothelial cells in clinical samples. They proposed that endothelial cell-derived factors contributed to the tumor growth initiated by CD133⁺ stem-like cells within vascular CSC niches [264]. However, the role of nestin is still unknown. shRNA-mediated nestin knockdown experiments in prostate cancer cells reduced migration in vitro and lung metastatic potentials in vivo [265]. Also in pancreatic cancer cell lines, such as AsPC-1 cells, artificially established nestin expression levels by transfection correlated with ability to migrate [266]. They showed that nestin expression levels were up-regulated by TGF β and hypoxia.

11.10.3.7 Wnt

In the MMTV-PyMT mouse breast cancer model, the CD24⁺CD90⁺ breast cancer stem cell (CSC) population constitutes roughly 3% of the whole tumor cells. The injected CSC through the tail vein showed prominent colonization in the lungs, in which periostin (POSTN) expression was induced in lung fibroblasts by CSC-derived TGF β . Given that POSTN induction was not detected in premetastatic lungs, postmetastatic interaction between CSC and fibroblasts is essential. They observed immune-positive POSTN staining in 17 of 23 (75%) auxiliary lymph nodes of breast cancer patients. Binding of POSTN with Wnt3a, which is capable of inducing HSC expansion (see above), promoted the Wnt signaling as judged by phosphorylation of the co-receptor for Frizzled, namely LRP6. In POSTN-KO mice, the positive Wnt signaling in the lungs was abrogated but primary tumor formation and CSC content were not altered [267].

11.10.3.8 Notch

A number of studies that tries to associate Notch with tumor progression are nicely summarized in reference [268]. For example, both Notch1 and Notch 2 were

expressed in the nestin⁺ primary glioblastoma multiforme cells in clinical samples, which were colocalized with endothelial cells expressing Notch ligands, such as JAG1 and DLL4. The stem cell-like features were evaluated by CD133 expression and neurosphere growth in a manner dependent on the Notch ligands [269]. Although Notch may serve as a tumor suppressor given the p21 induction, the biological outcome depends on the cellular context as observed in ROS-induced keratinocyte transformation in which Myc contributes to metabolic activation for cell growth [270].

11.10.3.9 CXCR4 and TLR4

See Chap. 13.

11.10.3.10 MSC and Tumor Cells

Human breast cancer xenografts of MDA-MB-231, for example, are abundant in endogenous (murine) MSC. Mixed implantation of MDA-MB-231 with human MSC from bone marrow augmented lung metastasis [271]. When tumor cells were plated back into in vitro culture from primary tumors generated by the mixture and metastatic lung foci, the latter turned out to be no longer metastatic. Those excellent experiments clearly indicate that MSC confers metastatic potentials in a reversible manner. An interesting question is where the conferment takes place. Based on the idea of <seed and soil>, both seed-producing primary and soil-cultivating metastatic microenvironments need to be assessed. This chapter focuses on the former. A set of cytokine assay of MSC in the absence or presence of MDA-MB-231 in 3-day culture revealed that CCL5 (also named RANTES) was up-regulated to 60-fold in a mixed culture-dependent manner and MSC was the major source. Ectopic expression of CCL5 in MDA-MB-231 induced neither growth advantage nor EMT-analogous morphology, but it certainly potentiated lung metastasis by fivefold. CCR5, the receptor for CCL5, is expressed in MDA-MB-231 but not MSC. We see bidirectional activation between MDA-MB-231 and MSC. MDA-MB-231 stimulates MSC by some way to secrete CCL5, which in turn activates MDA-MB-231, expressing CCR5. CCR5-activated MDA-MB-231 is supposed to transmigrate through vasculature of primary tumor at first.

11.11 Intratumor Heterogeneity and Origin of Metastasis Clones

11.11.1 Clonal Divergence

Whole exome sequencing of clinical tumor samples including those from spatially distinct regions of primary tumors and metastatic lesions has been performed in both pancreatic and renal cell carcinomas. For example, the well-known VHL mutation of renal cell carcinoma was detected in all the samples but loss of function mutations of a subset of genes such as PTEN was observed in a manner of spatial heterogeneity, i.e., different patterns of mutations in different regions within a single tumor whether primary or metastatic [272]. Therefore, metastatic signatures could be determined but the secondary i.e. metastasizing tumor cells by themselves are still under-branching genetic evolution (Fig. 11.5).

Detailed spatiotemporal analyses of genetic alterations in pancreatic cancers from two independent groups provided similar results [273, 274]. For example, parental clones had driver mutations (mutations that can promote tumorigenesis), such as those in K-Ras, p53, and/or SMAD4, as well as many passenger mutations (mutations are unlikely to promote tumorigenesis) [275]. One patient showed that the parental clone had 49 founder mutations and 6 and 22 additional mutations in the lung and liver metastasis, respectively. Those additional mutations were heterogeneous, actually present in the primary tumor and may confer progressive phenotypes to the tumors in an organ-specific manner.

In a murine model of prostate cancer, a long latency of 19 weeks is required before the advent of adenocarcinoma from precancerous prostate intraepithelial neoplasia (PIN) that is developed in prostate-specific PTEN deletion but the mice can survive over 48 weeks (see also Chap. 12) [275, 276]. The TGF β -SMAD4 signaling, as represented by increased expression levels of SMAD4, gives a constraint against tumor progression in PIN, which is unleashed by additional deletion of SMAD4 to result in adenocarcinoma in 11 weeks and death of all the mice by 32 weeks. The same author also showed that mutations of PTEN and SMAD4 contributed to the prognosis of the prostate cancer patients [275].

Genetic alterations can occur not only step-by-step but also all at once as exemplified in chromothripsis as described in Chap. 10 and this may accelerate the tumor progression after clonal selection.

The heterogeneity not only in genetic mutation but also expression level represents the clonal characteristics. For example, there have been many studies, clinical and subclinical, on transcriptional profiling that may differentiate the primary tumor cells from those in metastatic lesions.

Joan Massague group showed an elegant experimental system to positively select highly metastatic clones to specific organs. In one experiment they injected human lung cancer-derived cell lines, called H2030 and PC9, by intracardiac inoculation into NOD-SCID mice and put the metastatic colonies in brain and bone individually back into tissue culture. This *in vivo* selection provided them

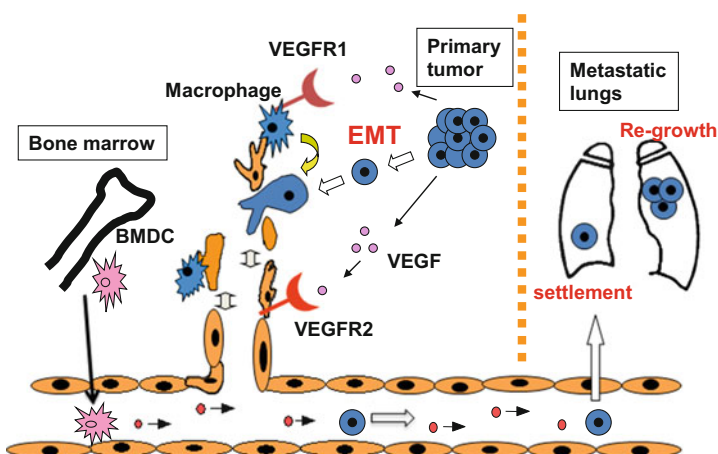
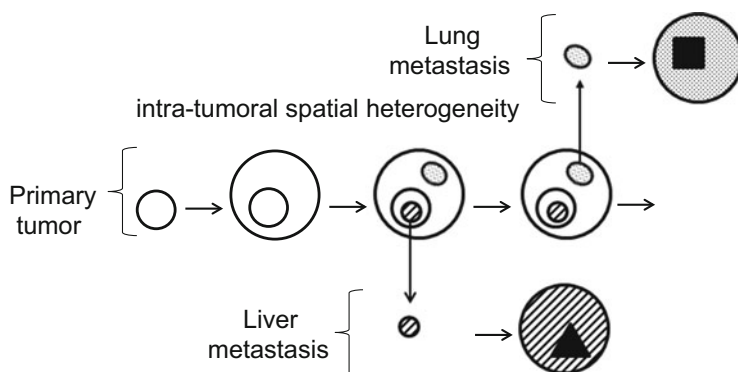


Fig. 11.5 Tumor cell heterogeneity. All metastatic clones originate from the primary tumor, but they continuously acquire mutations even at the metastatic sites

with clones with 100% metastatic potentials to each organ. Subsequent transcriptome analysis of brain-metastasizing clones revealed up-regulation of TCF4-induced HOXB9 and LEF1 expression [277].

11.11.2 EMT

Interconversion between polarized epithelial cells and mesenchymal cells plays an important role in embryonic development that requires highly programmed cell migration within the body. In 1982 Garry Greenburg and Elisabeth Hay showed loss of epithelial properties in a variety of polarized epithelial cells such as corneal

epithelium and acquisition of migrating ability in an in vitro collagen gel culture [278]. In the first step in cancer metastasis, epithelial tumor cells need to detach from the primary tumor and migrate or invade towards tumor vessels to make an entry into circulation. By utilizing gene microarray analysis of mouse mammary tumor cell lines with distinct properties, such as extravasation-defective and metastatic colonization-defective, etc. Robert A. Weinberg group identified the transcription factor Twist common to those two defective cell lines as well as to the highly metastatic cell line 4T1 [279]. Twist had been known as a master regulator of mesoderm specification in *Drosophila*. An siRNA-mediated knock down of Twist in 4T1 cells resulted in their poor ability to intravasate and eventually metastasize to the lungs. Loss of E-cadherin was observed as one of the effects caused by Twist. Although it can serve as a good indicator for EMT, an add-back expression of E-cadherin failed to revert the Twist-induced EMT.

Detailed cell behavior analysis with organotypic cultures, in which isolated mammary epithelium is embedded in 3D Matrigel, revealed that conditionally induced Twist1 caused individual cell dissemination as early as 24 h [280]. They showed three interesting points: (1) It was reversible upon turn-off of Twist1; (2) Twist-positive cells did not affect negative cell behaviors in the adenovirus-assisted mosaic experiments, i.e., cell-autonomous; (3) Although disseminating cells appeared embryonic mesenchymal cells, dissemination was rather abrogated by complete loss of E-cadherin and disseminated cells still retained epithelial identity. Twist1 appears to activate epithelial cell motility rather than mesenchymal cell fate.

As discussed in Chaps. 9 and 12, EMT is critical in metastasis and the relationship between CSC and metastasis has been studied extensively [281]. The linkage between EMT and CSC was proposed by the experiment in which forced expression of Twist in nontumorigenic immortalized human mammary epithelial cells gave CD44^{high}-CD24^{low} breast cancer stem cell phenotype [282]. We should be aware of the fact that one of the CSC criteria is the ability to grow in suspension as nonadherent mammospheres and the culture condition uses a black box chemical supplement called B27 potentially giving different experimental results [283].

Currently several transcription factors are known to be involved in EMT, including Twist, Snail, and ZEB1 [279, 284, 285]. Overexpression of Brachyury in human breast cancer cell MCF7 induced EMT phenotypes, such as invasiveness and secretion of cytokines, including IL-8, CCL5, and IL-6 [286]. IL-8, in turn, activated Brachyury to induce IL-8 and IL-8R, establishing an autocrine loop. Anti-IL-8R blocking antibody attenuated the invasiveness in vitro suggesting an essential role of IL-8 in Brachyury-provoked EMT. The discovery of the homeobox factor Prrx1 as an EMT inducer dissociated EMT from stemness [287]. TGF β could induce Prrx1. Intravenous injection of BT-549 breast cancer cells expressing Prrx1 induced lung metastasis only when Prrx1 was silenced by shRNA-mediated lentivirus. It also was true in orthotopically implanted BT-549 cells with subsequent lymph node metastasis. Prrx1 down-regulation resulted in increased expression of CD44, whereas that of Twist in decreased CD44.

Single-cell gene signature analysis of normal mammary cells and breast cancer cells whether in the primary region or metastatic sites revealed that metastasis-initiating cells expressed the pluripotency genes including OCT4 and SOX2 as well as EMT markers, such as Twist1, which were shifted to growth-promoting genes in high-burden metastatic cells, such as Myc and CDK2 [288]. A CDK inhibitor dinaciclib was effective in PDX breast cancer models (see Chap. 12).

I have no intention of stating the details of all the factors involved in EMT. The important point is what governs their activity in tumors. I introduce two mechanisms.

The first is hypoxia in tumor tissues. Hypoxia-stabilized HIF-1 α directly binds the Twist promoter. Hypoxia promoted EMT, which could be reversed by an siRNA-mediated knockdown of Twist [289].

The second is inflammation-induced stabilization of short-lived transcription factors such as Twist and Snail. IL-6 stimulates activation of CK2, which phosphorylates Twist preventing its proteasomal degradation [290]. While the half-life of endogenous Twist is roughly 70 min, the up-regulated Twist protein levels by IL-6 are sustained over the 4 h in lung cancer cell lines, such as A549. TNF α -NF κ B signaling induces (1) expression of CSN2 that mediates Snail stabilization and (2) up-regulation of Twist. While the half-life of Snail is 25 min, TNF α treatment can sustain the up-regulated Snail protein levels for the TNF α treatment period of up to 8 h in a manner sensitive to inhibitors of NF κ B (Sanguinarine) but not of mTOR (Rapamycin), p38 (SB-203580) or MAPK (PD98059).

In a xenograft model of MDA-MB435, concomitant administration of LPS enhanced lung metastasis, which could be abrogated by knock down of Snail.

NF κ B activation oscillates when induced by TNF α , individually by MyD88 or TRIF [291]. However, LPS elicits both MyD88 and TRIF signaling to have stable NF κ B activation.

Stable overexpression of Snail in HT29M6 human colorectal cells induced Sp1 transcription factor responsible for up-regulation of NKG2DL, such as MICA and ULBP1 (see Fig. 1.3 in Chap. 1) [292]. Therefore, Snail-mediated EMT renders the cells more sensitive to killing by NK cells with activated NKG2D.

11.11.3 HSC Niche Analogy: EphA4 versus EGFR-CSF1R Loop

Quantitative proteomic profiling of membrane-associated proteins in human immortalized mammary epithelial cells that were forced to undergo EMT by Twist, CD90 (also known as Thy1) and EphA4 were found to be up-regulated [293]. The corresponding counter-receptors, Mac1 (CD11b–CD18) and ephrins, respectively, were expressed in tumor-associated macrophages (TAM). The binding between those receptors and counterreceptors establishes a niche-like microenvironment by cell-cell adhesion. EphA4 elicited, in a tyrosine kinase

activity-dependent manner, a PKC δ -mediated NF κ B activation, which not only confers resistance against apoptosis on the tumor cells but also induces production of IL-6, IL-8, and GM-CSF. In their experimental system, the proposed loop of CSF1-EGFR in tumor cells and CSF1R-EGF in macrophages was not appreciated.

11.12 Concluding Remarks

Tumor microenvironment fosters and maintains not only tumor cells that are growing and in quiescence but also a variety of stromal cells of both local and bone marrow origins. Depending on the context, tumor cells are embedded in areas with angiogenic responses and degrading extracellular matrix or may be in niche-like compartments analogous to those of HSC.

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Chapter 12

Whole-Body Matter

12.1 Overview

In 1786, John Hunter observed pulmonary lesions as a metastatic manifestation from a tumorous disease in the femoral bone, which turned out to be a rare disease: osteosarcoma (see Table 9.1 in Chap. 9). This raised the curtain on metastatic diseases. The disease spreads from the leg to the chest as a whole-body matter. Here I will discuss cancer from the standpoint of inflammation, which is basically a whole-body matter with inevitable immunological reactions.

“Metastasis”, an organ-to-organ movement, of inflammation before “metastasis” of tumor cells is the basic idea of premetastasis, which will be discussed in Chap. 13. An advent of tumor cells within a given tissue is called carcinogenesis and that from different tissues, including the primary site, is called metastasis.

Inflammatory Carcinoma of Breast

There is a clinical term “inflammatory carcinoma of breast.” This type of breast cancer is manifested by edematous swelling, peau d’orange (redness of the skin), pain, and local hotness and therefore has four pathological features of inflammation proposed by Celsus (see Chap. 1). The essential pathology is tumor thrombus in the lymphatic ducts around the breast.

We cannot discuss the issue of cancer-associated inflammation unless we see cancer as a part of the whole-body where other organ(s) distant from the cancer tissue, including regional lymph nodes make interplay with the cancer through molecular and cellular elements. To study cancer from the view of whole-body, we need in vivo animal models. Well-known systems are summarized in the text. Depending on the context, cancer cell challenge to the host is rejected or gives rise to cancer development. Both are useful to study the mechanisms.

Here is an example of an old but educational experiment by Antonella Stoppacciaro in 1993 [1]. Murine colon cancer cells C26 derived from BALB/c mice chemically treated with N-nitroso- N-methylurethane can be cultured in vitro but also transplantable in syngeneic mice in which a minimum dose of 3×10^4 cells per mouse was required. Readers can recognize that tumor transplantation needs suitable genetic context and cell dosage. G-CSF-releasing C26 (90 pg/ml G-CSF per 10^6 C26 cells per 48 h in vitro) failed to grow in nonirradiated mice but did grow in sublethally irradiated mice at 600 rad. Genetically immunocompromised mice, such as SCID and sublethal irradiation to impair hematopoietic cells required for rejection often are used in transplantation experiments. Detailed immunohistochemical and morphological analysis of C26/G-CSF tumors that grew up to 15 mm in 30 days followed by regression revealed that neutrophils appeared in 15–20 days followed by macrophages in 20–30 days and then both $CD4^+$ and $CD8^+$ T cells. Simple depletion of neutrophils by anti-Gr1 (Ly6G) RB6-8C5 resulted in reduced T cells and inhibition of tumor regression. $CD8^+$ T-cell depletion also induced attenuation of tumor regression. Thus, the early-coming neutrophils are in favor of the late-coming cells.

12.2 Mouse Models

12.2.1 *Terms in Genetics*

“Syngenic” means genetically identical mice.

“Congenic” indicates only one locus difference between two mice. Frequently used CD45.1 and CD45.2 are functionally identical but genetically different giving an experimental situation where adoptive transfer is not rejected due to the absence, although not perfect, of alloreactivity by recipient $CD8^+$ T cells and transferred cells can be distinguished from the host cells [2].

“Allogenic” means of same species.

“Xenogenic” means engraftment between different species, i.e., from human to mouse.

Xenograft, allograft, syngraft, and autograft mean transplantation between different, same species, genetically identical, and self, respectively.

For example, C57BL/6 mice are intrasplenically injected by murine colon cancer cells SL4 of C57BL/6 of syngeneic origin. This results in liver metastasis in 14 days. Transplantation of the asbestos-induced murine malignant mesothelioma cells AB12 to the flank of BALB/c mice and Lewis lung carcinoma (LLC) cells to that of C57BL/6 mice also are syngenic, and the formed tumors are called flank tumors (ectopic syngraft). Intracardiac injection of human breast cancer cells MDA-MB-231 (an established cell line from metastatic pleural fluid) labeled with eGFP into C57BL/6 mice causes fluorescence-mediated quantification of bone-metastasizing cells in 24 h (ectopic xenograft).

12.2.2 Metastasis Models

Listed below are well-utilized mouse metastasis models.

12.2.2.1 4 T1 Cells

4 T1 cells are transplantable murine mammary tumor cells spontaneously arising from BALB/c mice [3]. They form tumors when injected to mammary gland and can spontaneously metastasize to a variety of organs, including lung, bone, and lymph nodes.

12.2.2.2 TS/A Cells

TS/A cells are spontaneously arisen mammary adenocarcinoma from BALB/c female mice and spontaneously metastasize to lungs [4].

12.2.2.3 MMTV-PyMT Model

Mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) transgenic mice display stages of autochthonous hyperplasia-adenoma-carcinoma and metastasis with high resemblance to human luminal breast cancer [5].

12.2.2.4 K14-HPV16 Mice Skin Cancer Model

The early region genes of human papillomavirus type 16 (HPV16) are expressed under the control of the human keratin 14 (K14) promoter [6]. The transgenic mice exhibit multistage epithelial carcinogenesis, i.e., epidermal hyperplasia by 1 month, hyperproliferative epidermis devoid of terminal differentiation where focal angiogenic dysplasia and infiltration of CD45⁺ leukocytes between 3 and 6 months, skin carcinoma in 60 % by 12 months with metastatic progression to regional lymph nodes in 30 %.

12.2.2.5 K5-SOS Mice Model of Skin Papilloma

Transgenic mice of the Ras activator Son of Sevenless under the keratin 5 promoter display spontaneously developing skin papillomas [7].

12.2.2.6 KPC Model of Pancreatic Cancer

K-Ras and p53 have genetic mutations in pancreatic cancers in more than 90 % and 75 %, respectively. K-Ras G12D and p53R172H (see Chap. 9) are conditionally expressed under the pancreatic and duodenal homeobox-1 (Pdx-1) promoter [8]. Pancreatic cancers manifest themselves at approximately 10 weeks with potential live metastasis. Chromosomal aberrations and centrosome amplification are observed.

12.2.2.7 RIP-Tag2 Mice in the C57BL/6 Background

Transgenic mice in which a highly vascular tumor develops in a pancreatic β -cell-specific manner by expression of the large T antigen of SV40 driven by the upstream region of the rat insulin gene [9]. Hypoglycemia by insulin-secreting tumors is treated by administration of a high sugar diet.

12.2.2.8 HPV16/E2 Mice in the FVB/n Background

Heterozygous K14-HPV16 transgenic mice were subcutaneously implanted with pellets that continuously release E2 in their dorsal back skin at 1 month of age [10]. Progression from premalignant intraepithelial neoplasia (CIN-1/2), high-grade dysplasia (CIN-3), and to fully transformed squamous cell carcinomas is observed.

12.2.2.9 HGF-CDK4 (R24C) Model Mice of Malignant Melanoma

CDK4 (R24C) mice are knock-in mice that express an activating mutant CDK4 incapable of binding to p16INK4a. HGF mice are transgenic mice expressing HGF under the metallothionein promoter. After backcrossing to the C57BL/6 background, both mice are mated to generate HGF-CDK4 (R24C) mice, which spontaneously develop primary autochthonous melanoma in the skin after painting with 7,12-dimethylbenz(a)anthracene (DMBA). Melanoma in those mice grows in a few months and spontaneously metastasizes to lymph nodes and lungs.

12.2.2.10 PTEN-KO Mice of Prostate Cancer Model

Prostate-specific PTEN deletion was achieved by crossing PTEN loxp/loxp mice with ARR2 Probasin-Cre transgenic mice called PB-Cre4 [11]. The Cre recombinase is under the control of the rat prostate-specific probasin (PB) promoter. The disease develops from intraepithelial neoplasia (PIN) with

9 weeks to invasive adenocarcinoma after 12 weeks followed by metastatic progression to distant organs, such as lymph nodes and lungs. The prostate cancer cells in both the primary tumor and metastatic tumors are positive in androgen receptor (AR), and regress under androgen ablation by castration at a few months in the invasive stage. However, they regrow 2.5 months after castration even if they remain AR⁺, which is similar to clinical prostate cancer.

12.2.2.11 PDX Models

Sliced tumors derived from surgical resections are subjected to implantation into immune-compromised mice, and, if transplantable, the tumors are passaged to other mice for maintenance of the patient-derived xenografts (PDX) [12]. The criticisms include that they are just approximation of the real course with poor understanding of selection pressure of engraftment and that difference from cell line-based models does not necessarily guarantee more reliable clinical relevance [13]. We, who routinely use nonimmunocompromised mice for metastasis experiments, are concerned with the immune deficiency that affects the biological outcomes.

To avoid rejection of graft, PDX models are based on mouse strains ranging from nude mice to NSG mice (see Chap. 9). Reconstitution of a human immune system in the mouse, called immunoavatar, sounds promising especially for the study of immune therapeutics.

12.2.3 *Orthotopic versus Ectopic Model*

Transplantation of tumor cells into their tissue of anatomical origin develop tumors in the normal place, i.e., breast cancer cells implantation to mammary pads (orthotopic) but not subcutaneous tissues (ectopic). Adenoviruses expressing Cre recombinase are intranasally administered to mice engineered to carry the Lox-Stop-Lox(LSL) K-Ras with activating mutation G12D to conditionally express the oncogene and subsequently to induce cancer in the bronchial epithelium in 8 weeks [14]. Removal of the transcriptional Stop results in K-Ras G12D expression. Injection of human MDA-MB-231 cells into the mammary fat pad of immune-deficient C.B-17 SCID mice (see Chap. 9 for immune-compromised mice) is called orthotopic xenograft.

Monitoring tumor growth is easy in ectopic models, but some tumors undergo necrosis during tumorigenesis. Although tumor growth evaluation is difficult in orthotopic models without sacrifice unless imaging studies by expensive equipment are performed, orthotopic tumors manifest more relevance to clinical course, such as invasion and metastasis, than ectopic tumors.

12.2.4 *Idea of Immune Privilege*

Allogeneic inoculation of P815 mastocytoma cells derived from DBA/2 mice into the anterior chamber of BALB/c mice resulted in intraocular tumors with continuous seeding throughout the body, but the metastasized tumor cells never grew [15]. A subcutaneous challenge of the same tumor cells, which usually generated flank tumors in tumor-non-bearing BALB/c mice, in those ocular tumor-bearing mice was rejected. In those ectopic allografts, the anterior chamber is called an immune privileged site.

Murine lymphoma cell EL4 was derived from C57BL/6 mice chemically induced by 9,10-dimethyl-1,2-benzanthracene (DMBA). Transfection of chicken ovalbumin cDNA into EL4 gave E.G7-OVA cells, which were rejected upon subcutaneous transplantation to syngeneic C57BL/6 but allowed to develop tumors when injected into the anterior chamber of the right eye. Interestingly, when the same tumor cells were subsequently challenged to skin, they were rejected [16]. Participation of CD11b⁺Gr1⁺ and CD11b⁺Gr1⁻ cells that accumulated in the chamber is assumed to inhibit the CD8⁺ cytotoxic T cells against ovalbumin. However, the precise mechanisms of immune privilege still remain uncovered.

12.2.5 *Cancers that Result from Inflammation*

In Chap. 10, I discussed cell transformation by referring to intrinsic factors, such as oncogenes and tumor suppressors. Those genetic alterations could happen in a single cell-based manner during the course of physiological activities, such as metabolism and DNA rearrangement. In addition, inflammatory responses could be causal events.

Well-known cancer-predisposing microbes of persistent infection include hepatitis B and C viruses in hepatocellular carcinoma and *Helicobacter pylori* in gastric cancer. Chronic inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, of unknown etiology are inflammation of apparent endogenous origin with predisposition to colon cancer. However, intestinal microbes could be involved in the carcinogenesis, because intestinal barrier disruption by ulcers and erosions allows their invasion. Inflammation-induced recruitment of leukocytes with ROS production is assumed to contribute to causal genetic alterations. Of note, complication of lung cancer is not known in chronic granulomatous disease (see Chap. 2) with an impaired ability of ROS generation due to Nox2 mutations. I introduce two nicely designed experiments to prove cancer development by persistent inflammatory conditions.

12.2.5.1 K14-HPV16 Transgenic Mouse Model of Skin Carcinogenesis

Lisa M. Coussens group found not only autoantibodies in the serum against HPV16 E7 oncoproteins, type I, II, and IV collagens, but also stromal depositions of immune complexes of IgG without B-cell infiltration during the epidermal hyperplasia stage before cancer development. The skin cancer development is dependent on the binding of the immune complexes to Fc γ Rs other than type II Fc γ R, because K14-HPV16 mice intercrossed with Fc γ -/- (see Chap. 2 for detail) exhibited negative effects on cancer development being virtually stalled at an early hyperplastic stage with decreased infiltration of mast cells and immature myeloid cells and less angiogenesis [17]. M1 cytokines (see below) were up-regulated in the skin. Mast cells express Fc γ RIII. Conditioned medium of Fc γ -deficient and proficient mast cells stimulated by HPV16-specific IgG contained VEGF-A capable of stimulating migration of HUVEC.

12.2.5.2 *Helicobacter Felis*/C57BL/6 Murine Gastric Cancer Model

In the first model of skin cancer, the immune response as represented by neutrophil infiltration transformed preexisting skin epithelial cells. In this model by Timothy C. Wang group, immune cells by themselves that infiltrate into gastric mucosa were transformed in the infected microenvironment to adenocarcinoma cells [18, 19]. Lethally irradiated male C57BL/6 mice were subjected to bone marrow transplantation (BMT) by bone marrow cells derived from C57BL/6 transgenic mice constitutively expressing β -galactosidase or chicken β -actin EGFP. β -galactosidase⁺ or EGFP⁺ cells with XY chromosome appeared in the post-apoptotic gastric mucosa 6–8 weeks after inoculation of *Helicobacter felis* in those BMT mice and were eventually transformed. Gastric mucosal injury by other methods, such as acetic acid administration, failed to induce this phenomenon. The up-regulation of CXCL12 and SCF in the infected mucosa was responsible for recruiting mesenchymal stem cells (MSC) from bone marrow that were capable of epithelial differentiation in vitro in the presence of soluble factors derived from the epithelial tissue.

12.3 Basic Issues that Need to Be Addressed

Leukocyte response is basically a whole-body matter. My definition of inflammation is principally mobilization of leukocyte and vascular permeability. Factors derived from tumor cells per se or in concert with surrounding cells do both. The idea is based on the extension of tumor field from a tumor tissue in the ascending colon, which will be described in Chap. 13, to a tumor-harboring whole organ,

colon as an organ, including seemingly normal sigmoid colon. Moreover, the tumor field extends from colon to the whole body, including bone marrow.

Primary tumor-secreted active molecules stimulate other organs in an endocrine fashion. The most sensitive organ is bone marrow, which releases leukocytes to other organs, including the primary tumor. Therefore, the tumor field is extended to involve simply the primary tumor, circulation, and bone marrow. Bone marrow-derived cell mobilization into the primary tumor is inflammation.

Given the factors involved in tumor progression, such as IL-6 and PGE₂, it is not surprising, but rather normal, that cancer patients often have fever (see Chap. 8). Why do tumor cells have mutations in growth or survival-promoting genes and not in key inflammatory cytokines, such as IL-1 and TNF α ? Maybe it is more favorable for tumor cells to adapt to flexible microenvironment rather than fixed state by genetic alterations of inflammatory cytokine genes.

1. What causes inflammation in tumor microenvironment?
2. Why do both tumor cells and leukocytes need to spread into other parts of the body by passive or active process?
3. Does education (stimulation) of cells take place in other place?
4. What are the driving forces?

12.4 Cancer Cachexia

Cancer cachexia is seemingly based on the interaction between cancer and host organs, especially adipose and muscle tissues. Upon malnutrition, mobilization of lipid from adipose tissue, glucose from liver, and amino acids, such as Gln from muscle, take place. Plasma lipoproteins that are generated after absorption from intestinal tracts are hydrolyzed by lipoprotein lipase, which is expressed in various organs, including adipose tissue, and localized in the luminal surface of endothelial cells after secretion to yield fatty acids (FA), which are then transported to adipose tissues for triacylglycerol synthesis and storage. Hormone-sensitive lipase (HSL) catalyzes the reaction to produce nonesterified FA from triacylglycerol. Therefore, signaling through the cAMP-PKA pathway that can activate HSL results in lipolysis, which is mediated by adrenergic agonists, such as epinephrine in physiological conditions.

Increased HSL expression in adipocytes for FA mobilization is observed in cachexia. Readers should remember that tumor cells prefer lipids as a source for their energy metabolism (see Chap. 10). In addition, purified lipid mobilizing factor from melanoma cells turned out to be zinc α 2-glycoprotein (ZAG) (Fig. 10.3 in Chap. 10). Given that the ZAG effect is mediated by β -adrenergic receptor (β AR) whose activation causes protein synthesis in and hypertrophy of skeletal muscle, it is likely that lipolysis precedes rhabdomyolysis in cachexia. Increased body weight is observed in ZAG knockout mice [20]. ZAG expression is regulated positively and negatively by PPAR γ agonists and TNF α in adipose tissues, respectively.

An early study in 1948 with gastric cancer patients who were administered with glucose via stomach tubes before liver sampling to measure glycogen showed that the patients failed to convert glucose to glycogen in liver, on which insulin injection has no effect [21]. In mice subcutaneously implanted with MAC16 colon cancer cells that manifested body weight loss in 10–12 days, there was correlation between weight loss and hepatic glycogen concentration [22]. ZAG also participated in the cGMP-mediated activation of hepatic adenylate cyclase in this process.

Protein degradation can take place in skeletal muscle by IL-6, TNF α , angiotensin II, LPS, proteolysis-inducing factor, and so forth. Increment of intracellular Ca²⁺, activation of NF κ B, and induction of the ubiquitin-proteasome pathway are involved in proteolysis of muscle proteins, such as myosin heavy chain.

Taken together, cachexia is caused by tumor-related factors that also are responsible for tumor-associated inflammation, including TLR4 endogenous agonists, such as FA (see Chap. 14), β AR agonists, IL-6, and TNF α .

12.5 Incoming and Outgoing Cells via Circulation

Intravasation of tumor cells takes place in vessels where perivascular macrophages are present. Like outgoing tumor cells by intravasation, incoming immune cells also have complicated mechanisms. In Chap. 11, I stated that VEGFR2-activated FAK with tyrosine phosphorylation at Y658 was responsible for vascular permeability. In confluent rat brain microvascular endothelial cell line GPNT transfected with VE-cadherin mutated at Y658, but not Y645, 731, or 733, failed to affect transendothelial migration of myelin basic protein antigen-specific T cells following ICAM1 activation by crosslinking signals through RhoA and MAPK [23].

In physiological conditions, monocytes make transendothelial migration at low basement membrane regions without changing the basement membrane. In this protease-independent condition, nuclear deformity restricts the migration. Given that leukocytes can deform their nucleus due to the lack of nuclear envelop protein laminin A/C that are generated from the same gene [24]. VEGF-induced Src activation changes the status of the VE-cadherin-mediated adherens junctions. A recent live-cell imaging study revealed that filopodia of approximately 1 μ m disrupt cell-cell endothelial junctions, which enlarges to 9 μ m, large enough for the tumor cell nucleus to pass. Of note, the process left no sign of basement membrane damage after extravasation is achieved. Interestingly, an immune-histochemical analysis reported that 47 % of 108 ovarian cancer samples lacked laminin A/V expression. Although laminin A/C is not required for cell division, its deficiency induced enlargement of nucleus from 18 to 29 μ m on average with aneuploidy accompanied by p53-dependent expression of p21 [24].

12.5.1 Immune attack

First I will review tumor-infiltrating lymphocytes. Autoantibodies against tumors are produced by B cells presumably in the draining lymph nodes at the early stages of carcinogenesis. In fact, autoantibodies against HER2 and p53 could be detected in the serum of breast cancer patients 150 days before the diagnosis [25]. Breast cancers also host infiltrating B cells often in combination with T cells and DC forming tertiary lymphoid structures found in nonlymphoid tissues. The cancer-activated B cells facilitate T-cell activation.

Antitumor immune responses by T cells are executed against tumor antigens as in the case of other foreign materials (see Chap. 2 for detail). Briefly, HLA class I-restricted peptides are processed in DC, which then activates naïve T to cytotoxic T cells. Readers remember that the most understandable oncogene BCR-ABL is derived from the endogenous proteins BCR and ABL but is structurally distinct from them by their pathological fusion. Therefore, BCR-ABL is a transformed self (see the idea in Chap. 6). Despite this clear circumstance, clinical vaccination trials could hardly detect BCR-ABL-specific CTLs. Transfected DCs with BCR-ABL RNAs in vitro were used as antigen-presenting cells to induce CTLs, which failed to recognize the BCR-ABL fusion protein epitopes. Mutated selfs may not serve as an immune-dominant antigen [26]. For example, clinical trials of anti-bladder cancer immunotherapy facilitate antigen-specific T cell activation by injecting a mixture of five synthetic peptides of nine to ten amino acids in length derived from corresponding five tumor antigens, including DEPDC1, MPHOSPH1, URLC10, CDCA1, and KOC1, all of which are highly expressed in cancers [27, 28]. A genome-wide cDNA microarray analysis with 27,648 genes of cancer cells after microdissection revealed overexpression of DEP (Dishevelled, EGL-10, Pleckstrin) domain containing 1 (DEPDC1) and MPHOSPH1 (M phase phosphoprotein 1). An siRNA-mediated knockdown of DEPDC1 or MPHOSPH1 in bladder cancer cell lines, such as J82 and UM-UC-3, resulted in growth inhibition in soft agar. Conversely, introduction of DEPDC1 but not MPHOSPH1 in NIH3T3 cells promoted cell growth. Therefore, irrespective of whether or not the gene of interest is sufficient for bladder cancer growth, they can serve as tumor antigens to activate CD8⁺ T cells to develop cytotoxic T cells (CTL) that attack bladder cancer cells expressing any of those. T cell receptor repertoire analysis is performed with cDNAs from peripheral blood monocytes from immunized patients. However, T cells find both physical and functional obstacles to limit their cytotoxic navigation close to tumor cells.

12.5.1.1 Evading Immune Attacks

Physical Obstacles

“Matrix Fibers” Physical Block

Herein, I introduce a nice method to visualize how T cells migrate around tumor microenvironment. Operationally resected human tumor samples were freshly embedded in agarose and sliced, on which Hoechst-labeled T cells of various conditions were overlaid [29]. Time-dependent analysis of tumors, such as lung cancer and colon cancer, revealed that T cells, whether CD4⁺ or CD8⁺, were much more abundantly found in the stroma than the tumor islets as monitored by fibronectin and EpCAM, respectively. Peritumoral dense fibers of extracellular matrix (ECM), such as collagen and fibronectin, which were possibly produced by tumor-associated active fibroblasts, were engaged in the T-cell displacement from the tumor, and T cells found their way in loose matrix region (called contact guidance) [30]. Activated T cells have poor expression in MT1-MMP. Integrin β 1 and β 2 are dispensable for migration within the stroma, because blocking antibodies against either of them failed to influence T-cell accumulation in the stroma. ConA-stimulated T cells failed to release FITC from FITC-labeled collagen of in situ collagen degradation assay.

In a xenograft model of tumor with and without enforced expression of CCL5, ratio of T cells found in the tumor islet was 20 % and 0.45 %, respectively.

“Endothelial Cells” Contact Failure

Generally tumor tissues are vascularized highly due to abundant VEGF expression from infiltrating macrophages and tumor cells. However, T cells poorly adhere to endothelial cells. For example, whereas VCAM-1 and E-selectin are expressed in endothelial cells in the peritumoral region, they are poorly expressed in intratumoral endothelial cells. This circumstance makes T cells distributed in the peritumoral region difficult to get access to tumor cells.

However, it does not necessarily exclude the interaction between those T cells and myeloid cells, macrophages, and tumor cells in hypoxic microenvironment as discussed below. It is noteworthy that not only tumor cells but also T cells are subjected to metabolic re-programming. Activated T cells use the same strategy as cancer cells (enhanced glycolysis rate, lowered rate of oxygen consumption in mitochondria, and addiction to glutamine, see Chap. 10 for details) to proliferate and migrate in hypoxic conditions, such as tumor microenvironment, lymph node, thymus, and wound with inflammation. A critical difference from tumor cells is that the process is reversible and the activated T cells eventually die. In a well established ex vivo model of antigen-stimulated T cells, in which primary T cells are activated by both anti-CD3 and anti-CD28, actively proliferating CD4⁺ and CD8⁺ T cells are obtained in a few days. The subsequent metabolomics analysis revealed up-regulated glycolysis and glutamine consumption and down-regulated

mitochondrial activities, such as oxidation of fatty acid and pyruvate [31]. By utilizing conditional knockout mice of either Myc or HIF-1 α , requirement of Myc but not HIF-1 α was demonstrated for the metabolic re-programming in the activated T cells.

It was reported that tumor-derived factors, such as TNF α , repress VCAM-1 expression. It is interesting that imiquimod, which blocks S100A9, reverses poor E-selectin expression and intratumoral Treg population (see Chap. 16).

Functional Obstacles

Old Cyclophosphamide-Mediated Tumor Rejection Model

Transplanted tumors are rejected in preimmunized but not naïve mice. In the latter case, those tumors are highly immunogenic and called regressors, and activation of T cells is followed by suppression by growing tumors. Most tumors can induce adaptive immune responses, but the presence or absence of rejection antigens does not correlate with the regressor phenotype and the immune response is not always destructive for tumors. Here are old experiments from the Thomas Blankenstein lab on tumor rejection by modulating stromal T cells and macrophages by a single injection of cyclophosphamide (Cy) without tumoricidal effects [32]. J558L plasmacytoma or Mc51.9 fibrosarcoma tumors transplanted in mice were rejected with necrosis within 3 weeks after Cy treatment, but those in nude mice or IFN γ R-KO mice were not, indicating dependency on T cells and IFN γ signaling in host but not tumor cells [33]. Macrophage infiltration as monitored by F4/80 and CD11b was independent of T-cell infiltration status. CD11b-positive cells isolated from tumors after but not before Cy treatment produced IFN γ without LPS stimulation, which was not observed with those from nude mice even when stimulated by LPS. However, T cells appeared to suppress IFN γ expression by macrophages in tumor-bearing mice, because T-cell depletion of them by intraperitoneal administration of anti-CD4 and anti-CD8 antibodies resulted in IFN γ production even before Cy treatment. Blood vessel destruction (see Chap. 11 for IFN γ effects on EC) preceded tumor necrosis. This shows that tumor eradication does not require antigen presentation by stroma cells and depends on T-cell-mediated production of IFN γ that actually induces endothelial disruption leading to hemorrhagic necrosis of the tumor [34].

Immuno-Modulator PD-1

To avoid hyperactivation of T cells by antigens, there are homeostatic controls by negative regulatory system. Programmed cell death receptor ligand 1 (PDL-1) (alternatively called CD274 or B7-H1) is expressed in T cells, macrophages, myeloid cells, and endothelial cells. PDL-1 binding to its receptor PD-1 (programmed death receptor-1) on activated T cells inhibits their proliferation. PDL-1 knockout mice showed hyperresponsive CD8⁺ T cells, and PD-1 knockout

mice displayed autoimmune diseases, such as glomerulonephritis (see Fig. 1.3 in Chap. 1).

PDL-1 is actually expressed also in tumor cells. Thus, the fine tuning of proliferation of activated CD8⁺ T cells by negative feedback is hijacked by tumor cells, which eventually results in immune suppression. Humanized anti-PD-1 monoclonal antibody nivolumab was shown to be effective for advanced melanoma (see Chap. 16 for details).

12.6 Incoming

12.6.1 *Tumor-Associated Macrophage Definition*

Macrophages are CD11b⁺F4/80⁺CD115 (CSF1R)⁺Gr1[−] population of cells and macrophages within tumor tissues are tumor-associated macrophage (TAM).

12.6.1.1 Overview

The term “tumor-associated macrophage” appeared in a paper in 1973 by Robert Evans. The author described it as macrophages within tumor tissues different from those in peritoneal cavity [35, 36]. He showed that intratumoral macrophages prepared by a method (i.e., treatment by trypsin and type I collagenase followed by serum-free culture for 24–48 h gave an effective purification of TAM that adhered and spread on culture dishes) from Chester Beatty Hooded rats implanted subcutaneously with a transplantable rat fibrosarcoma cells HSN, which is syngeneic for the rat and chemically induced by benzopyrene, displayed growth inhibitory activity against the HSN cells co-cultured in vitro, which was dramatically enhanced when TAM was treated by LPS, whereas the LPS by itself had no effect. Distant from the primary tumor, peritoneal macrophages from the same animal also showed growth inhibitory activity especially within 48 h from the start of the co-culture.

Those interesting experiments more than 40 years ago cast at least three essential questions even to recent tumor biologists:

1. First, where does TAM come from?
2. Second, is TAM growth-inhibitory or -stimulatory?
3. Third, how do primary tumors influence distant macrophages?

I will try to answer those questions.

12.6.1.2 Origin of Tissue Macrophages

Peripheral blood monocytes, which are $CD11b^+F4/80^+$ (mouse) or $CD11b^+CD11c^+CD14^+$ (human) cells with bean-shaped nucleus, released from bone marrow, are the precursors of tissue macrophages. The laboratory of Dan R. Littman showed that mouse monocytes consist of two subsets: $CX3CR1^{low}Gr-1^+CCR2^+$ subset and $CX3CR1^{high}Gr-1^-CCR2^-$ subset. Both are $Ki-67^-$ and $MHC\ II^-$ [37]. These correspond in humans to $CX3CR1^{low}CD14^{high}CCR2^+CD11b^{high}CD11c^{high}CD62L^+$ subset and $CX3CR1^{high}CD14^{low}CD16^{high}CD11b^+CD11c^+CD62L^-$ subset, respectively. The adoptively transferred $CCR2^+$ subset of cells isolated from CD45.1 mice into congenic CD45.2 mice disappeared in 24 h from the whole-body except spleen and therefore is short-lived in physiological conditions, but they invade into peritoneum by $CCR2$ -dependent migration when thioglycollate (TGL) was injected there to cause inflammation and become $F4/80^+$ inflammatory macrophages.

Conversely, transferred $CX3CR1^{high}$ subset of cells were long-lived, migrated in a $CX3CR1$ -dependent manner, and stayed in various organs, including blood and lungs, as $F4/80^{high}$ resident macrophages. This population of cells also is reported to be recruited during the repair phase of ischemic myocardium experiments and to express VEGF to promote healing [38].

Blockade of blood supply by coronary artery ligation induces cell death in cardiac smooth muscle cells. This recruits neutrophils within 1 day, which is followed by predominance of $Ly6C^{high}CCR2^+$ monocytes during the inflammatory phase of days 1–4 when $CCL2$ and $CX3CL1$ expression is up- and down-regulated, respectively, in the cardiac tissue and that of $CX3CR1^{high}$ monocytes after 5 days. In 15–20 days, monocytes and macrophages disappear whether by dying or emigrating from the infarct tissue. $Ly6C^{high}$ and $Ly6C^{low}$ monocytes failed to accumulate in $CCR2$ -deficient mice in the inflammatory phase and $CX3CR1$ -deficient mice in the repair phase, respectively. While $CCR2^+$ monocytes produce $TNF\alpha$, $CX3CR1^+$ monocytes secrete VEGF. The accumulation of $CX3CR1$ monocytes can be blocked by clodronate-loaded liposomes, which results in decreased collagen deposition and angiogenesis.

One of the critical factors for macrophages to accumulate in the primary tumor is $CCL2$ (alternatively called MCP-1) produced by the tumor. Stimulated myeloid cells as represented by $CD11b^+Ly6C^{high}CCR2^+$ blood monocytes derive from bone marrow before settlement as macrophages not only in the primary site but also in other parts of the body.

The TS/A model is a mammary tumor cell line of spontaneous occurrence capable of spontaneous lung metastasis. The transplanted tumor contained heterogeneous $CD11b$ populations with combinations of $Ly6C$, $MHCII$, $Ly6G$, and so forth. As the tumor enlarges, a $Ly6C^{low}MHCII^{low}$ population increases up to 60 % and is preferentially localized in hypoxic areas. Therefore, phenotypic markers on TAM can change in both temporal and spatial conditions.

What Is TGL?

Thioglycollate (TGL) has been widely used to induce sterile acute inflammation in, for example, peritoneal cavity. TGL is an irritant consisting of a variety of nutrients for bacterial growth, including proteose peptone, agar, beef infusion, and sodium TGL. When injected into the peritoneal cavity of mice, it induces neutrophil emigration peaked at 6 h, which is followed by cell death of the tissue, presumably releasing a variety of alarmins. Macrophage accumulation is peaked at 3 days. Discrimination of leukocytes in the peritoneal lavage fluid (PLF) was performed by their morphological examination by Wright staining. (Potentials of peritoneal macrophages to respond a combination of LPS and IFN γ to secrete TNF α and IL-12 were peaked at 4 and 2 days, respectively). For macrophages converted from those mobilized monocytes to migrate across the peritoneal membrane, in which mesothelial cells are underlined by basement membrane consisting of collagen IV and laminin, activated MMP9 on macrophages by plasmin is involved in the degradation of collagen IV. In fact, accumulation of macrophages was observed in the peritoneal tissue incapable of migrating across the barrier in plasminogen knockout mice intraperitoneally injected with TGL [39]. Given that TGL-induced macrophage recruitment is reduced by 44 % in MMP9 knockout mice and that CCL2 enhanced migration through Matrigel of macrophages that were proficient but not deficient in MMP9, CCL2-dominant and the plasmin-MMP9-dominant but still sensitive to CCL2 pathways for macrophage migration across peritoneal barrier appear to exist [40].

12.6.1.3 M1 and M2 (Fig. 12.1)

Mirroring the Th1 and Th2 system in T cells, macrophages are conceptually categorized to M1 and M2 phenotypes as two extremes in a spectrum [41, 42]. Th1 cytokines, including IFN γ , TNF α , and GM-CSF, produced from CD4⁺ type 1 Th cells, and LPS cause classical activation of macrophages to M1, which then express TLRs, NOS2, NO, ROS, TNF α , IFN γ , MHCII, IL-6, IL-12p40, and IL-1 β . Th2 cytokines from CD4⁺ T cells, such as IL-4, 10, 13, 21, and 33, induce alternative activation to M2 macrophages, which express arginase-1, TGF β , and VEGF resulting in immunosuppression and angiogenesis. The MHCIIlow population of cells showed the M2 markers, such as arginase-1 (Arg-1) and IL-4R α , were poor at DQ-Ovalbumin processing as antigen-presenting cells. Also in other model systems, such as 4T1 mammary tumor, which can metastasize from the primary mammary sites to other distant organs and 3LL, both M1 and M2 macrophages were observed.

CX3CL1 (alternatively named fractalkine) is unique among chemokines in that it is a transmembrane protein. Binding to its receptor CX3CR1, it mediates cell

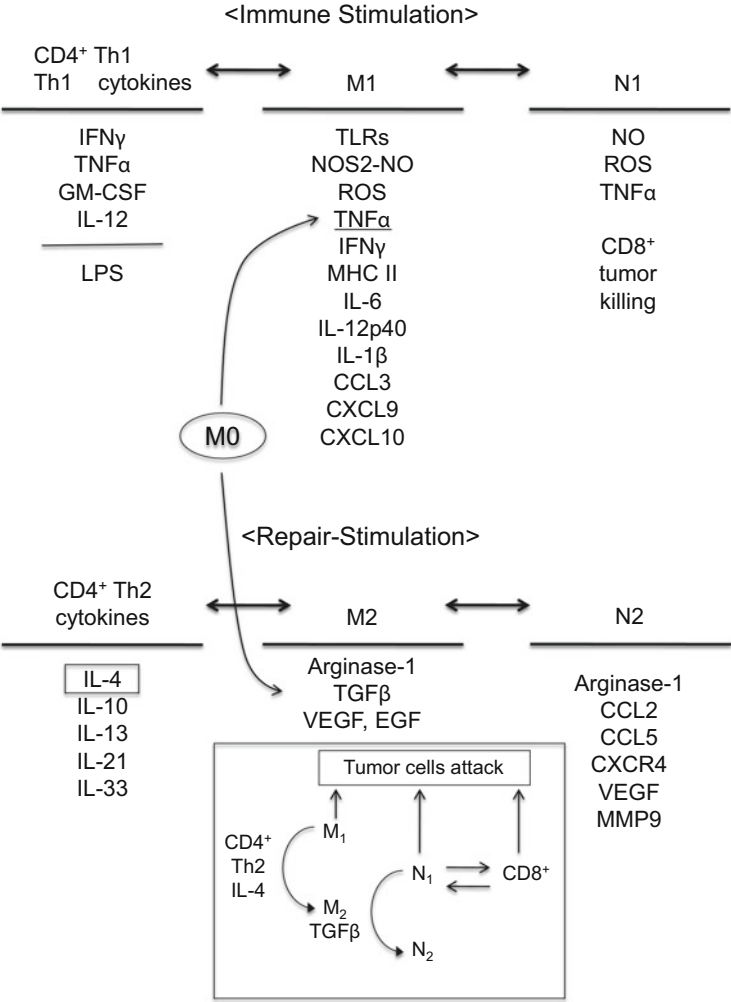


Fig. 12.1 Phenotypic switches in leukocytes. Numbers 1 and 2 are given not only to T cells (Th) but also macrophages (M) and neutrophils (N) with regulatory cytokines for each

adhesion. Upon shedding by ADAM10 and ADAM17, CX3CL1 is released. While CX3CL1 is produced in endothelial cells, CX3CR1 is expressed in monocytes, macrophages, activated platelets, and a variety of cancers, including colon cancer, breast cancer, and prostate cancer.

In a murine SL4 colon cancer model in which intrasplenic injection results in liver metastasis, CX3CR1 was abundantly expressed in F4/80⁺ TAM in metastatic lesions [43]. Liver metastasis was suppressed in CX3CR1 knockout mice with significantly decreased expression of VEGF and TGF β . In CX3CL1 knockout

mice, bone dissemination of human MDA-MB-231 breast cancer cells expressing CX3CR1 24 h after intracardiac injection was decreased by more than 70 %. Reciprocally, MDA-MB-231 cells expressing the mutated CX3CR1 at Y14F with a 100-fold diminished affinity to CX3CR1 gave the similar consequences [44]. Of note, the CX3CL1-CX3CR1 axis is involved in other disorders, such as Crohn's disease and cardiovascular disease [45].

12.6.1.4 The Question of Whether or Not TAM Is Proliferating

In the TS/A tumor system, TAM was Ki-67-negative, whereas CD11b⁺Ly6Chigh monocytes were Ki-67-positive. Although Ki-67 is expressed at ribosomal RNA synthesis sites in nonproliferating cells and therefore false-positivity could be observed, its absence indicates no proliferation. However, here are two interesting experiments to show that the TAM situation is not so simple.

The properties of macrophages in Th2 responses to parasites are discussed as follows. Analogy between tumor and parasite has long been discussed. Ly6ChighGr1intermediate cells and Ly6C⁻Gr1⁻ cells are potential precursors for F4/80⁺ macrophages in inflammatory and resident situations, respectively. Both can be suppressed by intravenous injection of clodronate-based liposomes. While thioglycollate induced accumulation of both Ly6ChighGr1high neutrophils and Ly6ChighGr1intermediate monocytes in the pleural cavity when injected intrathoracically, little accumulation of those types of cells were observed in nematode *Litomosoides* sigmoiditis infection [46]. However, expansion of F4/80⁺ resident macrophages was observed, which was not affected by clodronate but significantly reduced in the host background of IL-4 knockout. Intraperitoneal injection of IL-4 increased the macrophage numbers. Whereas 38 % of pleural cells were Ki-67-positive and 6 % were BrdU-positive under a 3-h pulse, blood monocytes were BrdU-negative. In CD45.1/CD45.2 congenic chimera mice that were generated by shielding the chest of CD45.1 mice under lethal irradiation followed by bone marrow transplantation with CD45.2 donor bone marrow cells, 30 % of leukocytes were of donor origin in peripheral blood, whereas only 3 % in pleural macrophages. Critically, TGL-induced pleuritis up-regulated the ratio to 30 %, but the parasite infection failed to do so with increased number of pleural macrophages. Those experiments clearly show that *in situ* proliferation of resident macrophages can take place in parasite infection in which IL-4 is necessary and sufficient.

Although activated macrophages secrete vasopermeability factors to induce inflammation, it is not accompanied by cell migration throughout the body. Permeability and cell migrations are not always concurrent in inflammation.

12.6.2 *T Cells Affect Macrophages*

Lisa M. Coussens lab, using the MMTV-PyMT mammary tumor model in combination with knockout conditions of RAG, CD4, and CD8, found that CD4 deficiency impaired both CD45⁺cytokeratin⁺ CTC and lung metastasis with no gross change in the primary tumor [47]. Elevated Th1 cytokines, such as TNF α , IL-6, and NOS2 (also called iNOS, inducible NOS), were observed in TAM in CD4-knockout conditions. Conversely, CD4⁺T cells expressed Th2 cytokines, including IL-4, IL-10, and IL-13 in MMTV-PyMT tumors. An in vitro stimulation of TAM isolated from CD4⁻knockout conditions by exogenous IL-4 induced the M2-type molecules, such as arginase-1 and TGF β , and EGF (Fig. 12.1). Lung metastasis was attenuated in the background of IL-4R inactivating mutation without changing the primary tumor. Thus, they demonstrated that IL-4-secreting CD4⁺ T cells directly stimulate a reciprocal growth-promoting interaction between CD11b⁺F4/80⁺Gr1⁻ TAM expressing EGF and CSF-1R and tumor cells expressing EGFR and CSF-1. This facilitated lung metastasis.

In a lung metastasis model of MMTV-PyMT mice crossed with Flt1tk^{-/-} mice in which the tyrosine kinase domain of VEGFR1 was knocked out, VEGFR1 kinase activity was found to be required spontaneous lung metastasis [48]. Application of an anti-VEGFR1 neutralizing antibody revealed that recruitment of CD11b⁺/F4/80⁺/Gr1⁻ macrophages to Met-1 cell metastasis in FVB mice was not affected but CSF1 expression downstream of VEGFR1 promoted lung metastasis.

12.6.3 *Myeloid-Derived Suppressor Cells*

Myeloid-derived suppressor cells (MDSC) are defined as CD11b⁺Gr-1⁺ cells that inhibit T cells.

12.6.3.1 Human MDSC

Myeloid-derived suppressor cells are mainly analyzed in mice (see below). Here is some information on human MDSC. In multiple myeloma patients, CD11b⁺CD14⁻CD33⁺CD15⁺ HLA⁻DR⁻ or low MDSC was significantly increased in bone marrow [49]. In CML patients in chronic phase with Sokal high-risk score, it was documented that levels of CD11b⁺CD14⁻CD33⁺ MDSC were higher than low-risk groups [50]. CD34 was expressed in 35 % of MDSC from clinical samples and CD11b⁺ cells expressed PDL-1 in 48 % in CML patients versus 23 % in control individuals. Colorectal cancer patients also have increased numbers of CD11b⁺CD14⁻CD33⁺ MDSC in both peripheral blood and intratumoral tissues, which correlates with tumor progression [51]. For a reference, human macrophages are CD16⁺CD68⁺CD115⁺CD163⁺CD312⁺.

12.6.3.2 Mouse MDSC Affect T Cells

Myeloid cells infiltrate in tumor microenvironment and play various types of protumoral roles, including inhibition of T-cell function. The idea was initially proposed in 1987 with metastatic LLC-bearing mice [52]. The ability of spleen cells to proliferate in response to T-cell mitogen Con A was suppressed by co-culture with bone marrow cells derived from tumor-bearing mice or normal bone marrow cell that were stimulated by the tumor conditioned medium obtained *in vitro*. There is a proposal of a cell population called myeloid-derived suppressor cells (MDSC) currently under debate [53]. It appears to be immature myeloid cells of heterogeneous population originally defined as CD11b⁺Gr1⁺ in murine cells capable of differentiating to macrophages, granulocytes, immature DC, osteoclasts, and even endothelial cells. Readers should remember that CD11b is integrin α M subunit and Mac-1 is α M β 2, alternatively CD11b⁻CD18 (see Fig. 1.2 in Chap. 1). Anti-Gr1 antibody, such as RB6-8C5, not only binds Ly6G but also its structural homolog Ly6C both of which are GPI-anchored proteins of unknown functions and have a discriminating property for subset of leukocytes. Ly6G expression is limited to granulocytes. The Ly6G equivalent to human is CD177 (HNA-2a, NB1, or PRV-1) capable of interacting with β 2 integrin (CD18) directly (Mac-1: CD11b⁻CD18, LFA-1: CD11a⁻CD18).

Thus, MDSC is subgrouped into CD11b⁺ Ly6G⁻ Ly6Chi monocytic MDSC and CD11b⁺ Ly6G⁺ Ly6Clo granulocytic MDSC. Most of the monocytic MDSC are F4/80⁺ and distributed diffusely throughout the tumor, such as B16 melanoma, whereas F4/80⁻ granulocytic MDSC is in the middle of the tumor. Monocytic MDSC makes a major contribution to T-cell inhibition via arginase-1 and NOS2.

L-arginine, essential for T cells, is depleted in the presence of arginase, which is generated by MDSC in response to IL-10, TGF β , or PGE2. Constitutive expression of COX-1 and COX-2 in 3LL tumor produces PGE2, which stimulates MDSC via EP4 to up-regulate arginase-1 [54]. TNF α and IFN γ are responsible for NOS2 induction and subsequent production of NO in MDSC [55]. Reacted with superoxide that also is generated by MDSC, highly toxic peroxynitrate is formed thereby efficiently inhibiting T-cell responses by nitration of TCR-CD8 [56]. In a murine skin allograft model, glucocorticoid induced NOS2 and subsequent production of NO in MDSC [57]. The experiment revealed that MDSC actively participates in allograft survival. S100A8/S100A9 complexes promote MDSC accumulation [58]. Conversely, S100A9 knockout mice are resistant to tumor challenge. Thus, S100A8/A9 is pro-tumorigenic.

Analysis of 40 gastric cancer patients showed that plasma S100A8-S100A9 levels were 93 ng/ml compared with 52 ng/ml in those in healthy donors and correlated with blood MDSC levels and reduced survival [59]. As judged by T-cell proliferation by mixing experiments with MDSC anti-S100A8-S100A9 and anti-RAGE antibody individually blocked the suppressive activity of MDSC. They authors assumed an autocrine loop of S100A8-A100A9-RAGE in MDSC.

In IL-17R-KO mice bearing mouse lymphoma EL4 transfected with the OVA gene, MDSC numbers decreased in both spleen and the tumor whose growth also was reduced [60]. Conversely, intravenous injection of adenoviruses expressing IL-17 followed by inoculation of the tumor resulted in increased numbers of MDSC in the spleen. The information indicated that IL-17 is necessary and sufficient for MDSC development in tumor-bearing mice. This is supported by other evidence that lung metastasis was abrogated after intravenous injection of 3LL in the background of IL-17-KO [61]. The authors also revealed that excess of IL-1 β activity in IL-1Ra-KO mice (see Chap. 8) stimulated $\gamma\delta$ T cells to secrete IL-17 resulting in enhanced lung metastasis. Anti-IL-1b neutralizing antibody augmented lung metastasis even in the background of IL-17-KO, for which increased numbers of Treg is responsible (see below for Treg).

MDSC can facilitate Treg development, thereby suppressing T-cell functions against tumor cells also in an indirect manner. The reported mechanisms are complicated but nicely reviewed by Lindau et al. [62].

Gene Therapy

Chimeric antigen receptor (CAR) strategy may be a “living drug.” To overcome the suppression of CD8⁺ T cells, recent technological advancement has given chimeric antigen receptor (CAR) strategy [63]. A specific antigen-recognition motif fused with the intracellular domain of CD3 ζ chain and CD137 (4-1BB, co-stimulatory receptor in T cells) signaling domain is transduced by lentiviral vectors in T cells to redirect their functions toward the antigen-carrying cells. One of the successful examples is CAR targeting the B-cell-specific antigen CD19 in chronic lymphocytic leukemia (CLL) [64]. Leukemic cells in CLL are predominantly resting with actively proliferating B-cell progenitor populations that are assumed to be exposed to antigen in both lymph nodes and bone marrow. Although Bruton tyrosine kinase inhibitor ibrutinib is promising, the CAR strategy also showed effective results. The CAR cells persistently appeared for 6 months in both the peripheral blood and bone marrow of the patients. Refractory B-cell ALL patients also were under successful trial [65].

Another potential gene therapy is intrapulmonary delivery of IFN β . The half-life of IFN β in blood is less than 60 min. To overcome this little bioavailability, adenoviral vector expressing IFN β was intranasally administered in K-RasG12D mutant mice infected with adenovirus expressing Cre recombinase to exert bronchogenic cancer in mice [66]. IFN β can stimulate both NK cells and CD8⁺ cytotoxic T cells to induce tumor lysis and up-regulate MHC class I expression. This gene therapy was not only effective in those mice but also interestingly suppressed the tumor growth of the rechallenged same tumor cells by establishment of immunogenic memory response.

12.6.3.3 Active Participation of MDSC in Tumor Progression

Differentiation of MDSC into osteoclasts and angiogenic endothelial cells has been observed, both of which actively facilitate tumor progression.

One of the prominent features of multiple myeloma is bone loss caused by enhanced osteoclast activity. In a murine model of multiple myeloma cell transplantation where PD-1 was increased in tumor-specific T cells, anti-PDL-1 antibody was effective for tumor rejection. This is what I explained as defense against immune attacks by inhibiting activation of T cells. In addition to this, MDSC from tumor-bearing LacZ transgenic mice revealed 14 times more potency to differentiate into mature LacZ-positive osteoclasts than non-tumor-bearing mice [67].

Breast cancers often cause osteolytic bone metastasis. CD11b⁺ Gr1⁺ MDSC isolated from MDA-MB-231 breast tumor-bearing mice could augment tumor growth when coinjected into the mammary pad of nude mice with MDA-MB-231 [68]. When MDA-MB-231 cells were injected into left ventricle and MDSC derived from breast tumor-bearing mice into tibial bone in athymic nude mice, the MDSC enhanced formation of osteolytic bone lesions. The experiments utilizing MDSC from GFP transgenic mice revealed that 46 % of GFP⁺ cells were tartrate-resistant acid phosphatase (TRAP)⁺ cells. Osteoclast-mediated bone destruction releases TGFβ, which stimulates growth of bone-metastasizing tumor cells and production of parathyroid hormone-related protein (PTHrP) capable of activating osteoclasts. This establishes a vicious cycle for bone loss by metastasis.

When CD11b⁺ Gr1⁺ cells sorted from spleen of mice bearing MC26 colorectal cancer cells were coinjected with MC26 cells into subcutaneous tissues, increased tumor growth rates and angiogenesis were observed [69], in which MMP9 derived from MDSC and VEGF from tumors played a significant role. Labeling of sorted MDSC with a vital dye 5-CFDA and anti-CD31 immunostaining revealed that MDSC differentiated into endothelial cells to promote the tumor growth. Endothelial progenitor cells from bone marrow are under debate, especially in humans [70] (see below).

12.6.4 Tumor-Associated Neutrophils (Fig. 12.2)

Tumor-associated neutrophils (TAN) are defined as CD11b⁺Ly6G⁺ cells with segmented nuclei found in nonnecrotic areas.

Levels of neutrophil infiltration in cancer and its association with patient prognosis of the cancer are dependent on the type of cancer and its stage. For example, analysis of 273 advanced human gastric cancers reported that 28 % showed 10 or more neutrophils over 20 nonoverlapping microscopic fields of 0.08 mm² each [71]. Neutrophils existed as 3–200 cells aggregates in the intratumoral regions and lymph nodes with tumor invasion. TAN turned out to be a prognosticator for higher

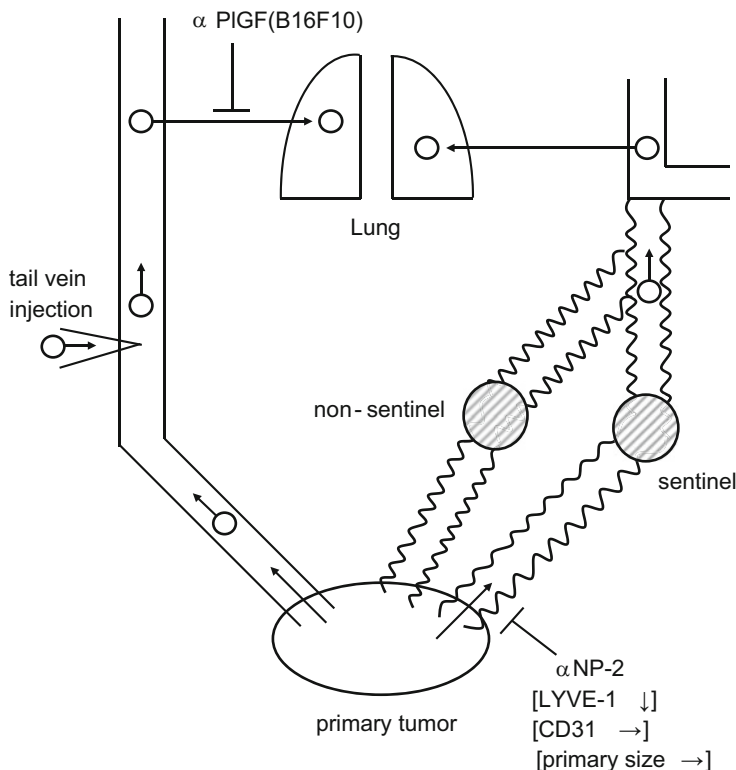


Fig. 12.2 Hematogenous and lymphatic dissemination. Primary tumor size depends on tumor angiogenesis but not lymphangiogenesis

survival rate. In 363 invasive human pancreatic tumors, 4 % revealed more than 10 neutrophils per 100 epithelial cells [72]. They were localized in intratumoral stroma. The uncommon presence of TAN is associated with more malignant pancreatic tumors of poor prognosis.

Detailed analysis by Zvi Gregorio Fridlender of TAN during the progression of flank tumors of AB12 in syngenic host mice revealed that roughly 1 % were TAN out of digested and dissociated LLC tumor cells [73]. TAN was found in peritumoral region at day 7 and intratumoral region at later stages of 14 days. The mRNA levels of CCL2, Arg-1, VEGF, and NOS2 were significantly increased in the sorted TAN compared with those in bone marrow neutrophils. TAN isolated from the tumor at early stages but not naïve mice displayed more tumor cell-killing activities than that at late stages due to the production of both H₂O₂ and NO, which mimics the phenotype of M1 macrophages. Thus, the idea of N1 vs N2 was proposed. In tumor progression, TAN becomes more and more mixture of N1 and N2. However, the same author documented that depletion of TAN at early stages failed to affect the tumor growth presumably due to the fact that N1 is localized in the peritumoral regions with poor access to the tumor cells.

What regulates the switch between N1 and N2? Interestingly, blockade of TGF β signaling by type I TGF β R kinase inhibitor SM16 or anti-TGF β neutralizing antibody induced more recruitment of TAN in the nonnecrotic areas without influencing the neutrophil counts in the peripheral blood in the same tumor model [73]. TGF β is derived from tumor cells and TAM, and appears to inhibit secretion of CXCL2, CXCL5, and GM-CSF capable of recruiting neutrophils, deinhibition of which by SM16 results in more tumor-killing neutrophil influx with the N1 property. Ly6G-mediated depletion of TAN in favor of N1 under this TGF β blocking condition led to significant reduction in CD8 $^{+}$ T-cell activation as monitored by CD137 (4-1BB). It is likely that N1 promotes CD8 $^{+}$ T-cell recruitment by secreting TNF α , CCL3, CXCL9, and CXCL10. Conversely, systemic depletion of CD8 $^{+}$ T cells, as monitored by more than 90 % loss from the spleen, resulted in abrogation of antitumor effect by SM16, suggesting that CD8 $^{+}$ T cells are effectors for antitumor activities downstream of the TGF β blockade with the TAN recruitment in between. Thus, TGF β inhibition induces N1 recruitment, which further allows CD8 $^{+}$ T cells entry into tumor tissues. Both N1 and CD8 $^{+}$ T cells participate in antitumor activity. In the absence of TGF β blockade, TGF β gives a favorable condition for N2 compared with N1. In this condition, depletion of TAN gave an opposite effect, i.e., increased CD8 $^{+}$ T-cell activation.

As opposed to TGF β , IFN β seems to skew differentiation toward N1. TAN recovered from transplanted B16 melanoma in IFN β -knockout mice highly expressed VEGF, CXCR4, and MMP9, which was suppressed when treated in vitro by IFN β , partially excluding the hypoxia-dependent VEGF expression [74]. When TAN from IFN β receptor KO mice were coinjected with the primary tumor cells into the secondary mice, they generated progressive tumors with active angiogenesis. Given that B16 melanoma transplanted to knockout mice of either IFN β or IFN β receptor grew faster with active angiogenesis and enhanced lung metastasis than those of wild-type and that VEGF-containing Matrigel subcutaneously implanted into IFN β knockout mice acquired more pronounced angiogenesis than that from wild-type mice, it is likely that IFN β presumably of host endothelial cell origin controls the excessive expression of those angiogenic factors in a homeostatic manner.

12.6.5 DC

The relationship between tumor and DC is not well understood. As we see in Chap. 2, classification of DC is complicated in both mouse and human. Here, I describe one nicely designed experiment to try to address this issue. Matthew F. Krummel group used a modified version of MMTV-PyMT breast cancer model in which mCherry to track tumor cells and the well-characterized chicken ovalbumin (OVA) to monitor the antigen processing were concomitantly expressed with the oncogene [75]. The OVA served as a self-antigen in tumor cells and specifically recognized by CD8 $^{+}$ T cells expressing the OT-I TCR (MHC class I-restricted,

ovalbumin-specific, CD8⁺ T). Adoptively transferred naïve GFP-labeled OT-I T cells to those mice were activated to migrate toward tumor cells but unable to eliminate the tumor cells. Majority of mCherry⁺CD45⁺ cells were CD11c⁺Gr1[−]F4/80^{high}MHCII^{high} and moderate CD11b, thus being qualified also as TAM but not MDSC, and abundant within the tumor. Their numbers did not change in the course of tumor development from hyperplasia to carcinoma. Cytotoxic activity of OT-I T cells isolated from the lymph nodes turned out to be high but that from the tumor was almost negative. They observed that the interaction between T cells and those DCs was more frequent within 5 μ M from the tumor cells. The isolated CD11c⁺MHCII⁺ cells, in which TLR4 was poorly expressed, were incapable of supporting proliferation of OT-I T cells, but the activity was restored to them by imiquimod or CpG capable of activating TLR7 and TLR9, respectively.

Thus, tumor antigens are processed by DCs that are engaged with T cells. However, the DCs fail to activate growth of CD8⁺ T cells.

Lymphoid Malignancy without Mutations or Nonmalignancy with a Mutation

Cytokine-dependent massive proliferation of blood cells may manifest as malignancy as represented by human large granular lymphocyte leukemia (LGL) with overexpression of IL-15 [76]. The IL-15 transgenic mice displayed LGL leukemia with NK cell phenotypes. Prolonged exposure of LGL with IL-15 induced up-regulation of aurora kinases A and B, centrosome abnormalities, and aneuploidy.

Conversely, the well-known mutation of melanoma, BRAF-V600E, was reported to be found in 64 % of 100 patients of Langerhans cell histiocytosis (LCH), a clonal disorder of still unknown etiology, which presents inflammatory lesions with infiltration of langerin⁺CD207⁺ DCs [77]. Conditional mice of BRAF-V600E under the CD11c promoter gave rise to an aggressive LCH-like disease.

12.6.6 Treg (Nonmyeloid Suppressor)

One of the major functions of Treg, previously known as suppressor T cells, is to limit overreaction of Th1, Th2, and Th17 cells in inflammation at the levels of transcription (see Chap. 2). IL-1 β -KO mice exhibited enhanced lung metastasis of intravenously injected 3LL cells (61), which was accompanied by increased numbers of Treg induced by TGF β (see Chap. 2). It was abrogated by anti-CD24-mediated depletion of Treg. In both human breast cancer specimens and murine MMTV-ErbB2 transgenic breast cancer model, Treg was found to be located in stroma adjacent to smooth muscle actin (SMA)⁺ myofibroblasts that secrete CCL5 [78]. In the tumor microenvironment, the CD4⁺CD25⁺Foxp3⁺ Treg cells are attracted by the CCL5-rich area by expressing CCR1, a receptor for CCL5, and produce RANKL [79]. RANK expressed in tumor cells signals through IKK α to repress for example maspin, an angiogenesis inhibitor (see Chap. 11). Reduced

gene dosage in RANK $\pm$ mice in the background of MMTV-ErbB2 impaired lung metastasis by 50 % as compared with RANK $^{+/+}$, and recombinant RANKL injection augmented lung metastasis by 1.8-fold. Similar to anti-inflammatory M2 and N2, negative cellular inhibitor Treg appears to contribute to tumor progression in tumor microenvironment.

12.6.7 Mast Cells

Mast cells differentiate from HSC-derived precursors CFU-mast cells to achieve terminal differentiation to CD11b $^{+}$ CD16 $^{+}$ CD34 $^{+}$ mature mast cells in the target tissues, including murine skin, respiratory tract, and intestinal tract.

In a variety of solid tumors, they are found in peritumoral as well as intratumoral regions, and clinical researchers have tried to correlate the mast cell information with prognosis depending on clinical circumstances. For example, analysis of 234 cases of invasive breast cancer revealed that presence of stromal mast cells adjacent to tumor cells as judged by immunostaining for MCP-6 (tryptase) correlated with low-grade tumors [80]. This is seemingly in agreement with the idea that mast cells may have antitumor effects. However, there has been conflicting information, suggesting a dependency on clinical circumstances [81].

In K14-HPV16 transgenic mice, a murine model of epithelial carcinogenesis, mast cells were found in all stages of tumor development, especially around the invading fronts, to secrete both of the mast cell-specific MCP-4 (chymase) and MCP-6 (tryptase) [82]. MCP-4 activates MMP9 to induce angiogenesis. Intercrossing with KITW/KITWV mice, in which c-Kit is mutated to cause mast cell deficiency of less than 1 % compared with the control, demonstrated that the angiogenic reaction was impaired. Mast cells also can generate VEGF and histamine for vascular permeability.

Local production of IL-10 by mast cells may give immune privilege to chronic bladder infection [83]. Allergic contact dermatitis with urishiol or 2,4-dinitrofluorobenzene is mediated by CD8 $^{+}$ T cells specific to those substances and aggravated in mice deficient in FcR γ (the common γ chain that is shared by Fc ϵ RI for IgE and Fc γ RIII for IgG) [84]. IL-10 production through FcR γ is thought to restrict the dermatitis.

MCP-6 also can induce recruitment of eosinophils possibly capable of killing tumor cells at least in vitro. Thus it is likely that functions of mast cells for tumor cells could be a double-edged sword.

There is a possibility that mast cells contain renin proteins as evidenced by cardiac mast cells, which could trigger angiotensin II generation (see Chap. 15) [85].

12.6.8 *Eosinophils*

Eosinophilic infiltration is frequently observed in parasitic infection and tumor. However, their roles are under debate. In chemical carcinogenesis model of methylcholanthrene (MCA)-induced fibrosarcoma in BALB/c mice, tumor development was inhibited in terms of MCA dosage, tumor incidence, and time required for occurrence in the background of IL-5 transgenic mice [86]. CCL11 (eotaxin, chemokine for eosinophils)-KO mice made a stark contrast with those mice and manifested aggressive tumor progression. Tumor-associated eosinophils correlated well with tumor suppression. The same authors claimed that MCA-induced fibrosarcoma cell line was directly killed by OVA-activated eosinophils that were stimulated PMA.

Subcutaneous injection of B16-F10 melanoma cells in syngeneic C57BL/6 J mice displayed eosinophilic infiltration in the necrotic areas in the center and capsule areas in the outer region, which was not affected by immune-depletion of CD4⁺ T cells [87].

12.7 *Outgoing*

12.7.1 *Overview*

The questions include how tumor cells migrate in the interstitial space and extravasate into circulation. Tumor cells can migrate in cluster consisting of more than a single cell in which cell-cell contact is retained. This collective migration is achieved by degrading ECM, such as collagen and fibronectin in a MT1-MMP-dependent manner [88]. During the movement in the interstitial space, EMT gives loss of cell-cell contact resulting in single-cell migration. There are two modes in single-cell migration. One is called mesenchymal migration or elongated cell migration in which colocalized MT1-MMP and $\beta 1$ integrins at cell-ECM contact sites provide the gliding force by degrading ECM. The other is amoeboid migration or round cell migration in which nonpolarized cells crawl through collagen fibers by adapting their morphology. The process neither accompanies protein degradation of fibers nor leaves long-lasting structural changes in ECM. This is similar to what I stated as contact guidance in T-cell migration in tumor tissues.

In addition to the role in eNOS (also called NOS3) inhibition in endothelial cells, stromal fibroblasts expressing caveolin-1 produce ECM, such as fibronectin, whose intratumoral alignment correlated with metastatic ability. The remodeled ECM by caveolin-1-expressing CAF enhances tumor cell elongation and directional migration, eventually giving high metastatic potentials to the tumor cells [89].

Circulating tumor cells exist in the form of single or in cluster before they penetrate through the endothelial barrier (see Fig. 9.4 in Chap. 9). The solid evidence should be real-time three dimensional (3D) imaging of the process.

Previously experiments were by plating tumor cells onto HUVEC monolayers to observe their transendothelial migration. For example, T24 human bladder cancer cells in spheroid were applied; endothelial retraction at the site of tumor cell-endothelial cell contact allowed the tumor cell spheroid to sneak through the endothelial gaps generated by the morphological changes. Recent advances in technology created a microfluidic platform method to visualize extravasation in real-time 3D, in which co-culture of HUVEC and normal human lung fibroblasts in hydrogel containing fibrinogen form 3D vascular network with lumen from 8 to 96 μm in diameter surrounded by collagen IV matrix and void of leakage as judged by 70 kDa dextran [90]. When MDA-MB-231 cells were seeded, time-lapse imaging showed that filopodial protrusions of 1 μm into subendothelial matrix were followed by enlargement of gaps to 10 μm as tumor cell nucleus transmigrates through the barrier. Fluorescent beads of 10 μm were trapped in vessel whose diameter size was smaller than that of beads but failed to extravasate, indicating that tumor cell extravasation is an active process. Neither disruption of VE-cadherin-mediated junction and nor dextran leakage were observed at the sites of extravasation. Efficiency of extravasation was higher in trapped than adhered cells to endothelial cells in vessels whose lumen size was larger than that of the tumor cells, and in clustered cells.

A variety of growth factors and chemokines are vascular permeability factors at the same time, including VEGF and $\text{TNF}\alpha$. $\text{TNF}\alpha$ at 2 ng/ml facilitated permeability of dextran by 2.1-fold and tumor cell extravasation by 2.3-fold. Of note, $\text{TNF}\alpha$ at 10 ng/ml caused apoptosis of endothelial cells resulting in rupture. Cell death of endothelial barrier also was observed in T24 spheroid plated onto HUVEC monolayers. I assume two types of transendothelial migration of tumor cells, single cells through reversible morphological alteration of endothelial barrier and cluster or spheroid through occasionally irreversibly damaged endothelial cells.

12.7.2 What Is the Destination of Extravasating Tumor Cells?

12.7.2.1 Interplay Between Two Organs

The evidence of HSC homing to their own niche in bone marrow has provided an idea that circulating tumor cells (CTC) may find their destination in bone marrow niche. Fundamental questions emerge about as to what extent CTC is similar to HSC and how to explain bone-sparing metastasis of many cancers.

In a NOD/SCID mice xenograft model in which a well-known human prostate cancer cell line PC3 was implanted into subcutaneous tissues, PC3 cells disseminated into the bone marrow in a niche size-independent manner as judged by osteoblast numbers impaired bone marrow transplantation supported by HSC engraftment. Lethally irradiated mice had HSC engraftment more inefficiently with bone marrow cells containing HSC purposely admixed with PC3 cells than without. Multiphoton imaging revealed that LSK-HSC prelabeled by fluorescent

nanocrystals and PC3 cells by GFP were colocalized in osteoblastic niche within five cell-distances apart from each other. PTH-induced expansion of the number of osteoblastic niche resulted in increased bone metastasis. Collectively, PC3 cells are likely to compete for osteoblastic niche with HSC [91]. The authors further showed interesting experiments of whether or not PC3 CTC after achieving homing can egress from the niche. Disruption of the CXCL12-CXCR4 signaling essential for HSC homeostasis by AMD3100 that can inhibit CXCR4 mRNA expression or G-CSF that can induce MMP2 and MMP9 to degrade CXCL12 actually mobilized PC3 cells from the bone marrow in primary tumor-resected animals.

Stem cell markers are different from cancer to cancer as stated in Chap. 9. In the case of prostate cancer, CD133⁺ CD44⁺ cells are proposed for repopulating ability. They observed that 0.86 % of PC3 cells of their in vitro culture were CD133⁺ CD44⁺, and this population expressed higher levels of CXCR4.

Although stem cell niches may be altered even if preexisted as in the case of bone marrow niches or newly generated if absent before, those experiments show that CSC can utilize preexisting bone marrow niche originally for HSC without cultivating “soil.” For cultivated soil in lungs, see Chap. 13.

12.7.2.2 Interplay Between Three Organs (Tumor, Marrow, Adipose)

Correlation between obesity and tumor progression has been shown in human. Stromal cells isolated from white adipose tissues (WAT) in GFP mice appeared in circulation when subcutaneously administered and could make an entry into the implanted tumors with B16 melanoma or LLC. Transplantation of WAT derived from GFP mice accelerated implanted tumor growth in non-GFP mice by the WAT stromal cells migrating into the tumor [92]. The phenomenon is interesting, but the precise determination of cell types and molecular mechanistic information were missing in those experiments.

12.7.2.3 Interplay Between Four Organs (Tumor, Marrow, CNS, Lung)

Does so-called chronic behavioral stress facilitate tumor metastasis? Let us think of this question from the view of inflammation. First of all, the stress activates both hypothalamic-pituitary-adrenal system and sympathetic nervous system (SNS) originated from central nervous system (CNS). In a mouse restraint system, in which mice are physically immobilized in restricted slot without wounding for a certain period of time, norepinephrine and corticosterone levels stably increased by up to 3.5- and 7.8-fold, respectively, in ovaries, spleen, and omentum while plasma norepinephrine levels were highly unstable [93]. In nude mice transplanted with human ovarian cancer cells into the peritoneal cavity, protein kinase A signaling caused enhanced angiogenesis and tumor growth in the tumors by inducing VEGF, MMP2 and MMP9 only when tumor cells expressed β 1 and/or 2 adrenergic receptor (β AR). This appears to be interplay between two organs, SNS and tumor.

The same group showed that SNS augments angiogenesis in primary tumor and metastasis without changing primary tumor growth in a different tumor model [94]. Now the interplay is established between four organs consisting of primary tumor, SNS, bone marrow, and lungs as a target of metastasis. Luciferase-tagged 66 cl4 breast cancer cells were injected into mammary pad for spontaneous metastasis or through the tail vein to force metastasis by circulation, and they tracked and quantified those cells in the body by *in vivo* imaging. A huge difference in the increased signals of lung metastatic foci between the spontaneous (38-fold) versus forced (2.7-fold) metastasis model under the same stress can be explained by SNS-dependent alterations in primary tumor microenvironment. The pro-metastatic signal originated from $\beta 2AR$ -expressing $CD11b^+F4/80^+$ macrophages in the primary tumor, which were increased in number by 53 % ($CD11b^+Gr1loLy6Chi$ mononuclear myeloid-derived suppressor cells “MO-MDSC” by 43 % and $CD11b^+Gr1hiLy6Clo$ polymorphonuclear MDSC “PMN-MDSC” with no significant change) and stimulated to up-regulate TGF β , Arg-1, MMP9, VCAM1, CSF1, VEGF, and so forth [94]. Bone marrow also is innervated by SNS and G-CSF-dependent egress of HSC and progenitors is requires SNS. Therefore, activated SNS by CNS stimulates both blood cells in primary tumor and bone marrow eventually causing βAR signaling from increased number of macrophages in the primary tumor. Both the increase in the number of macrophages in the primary tumor and the lung metastasis were abrogated by a selective CSF-1R inhibitor GW2580 that was shown to selectively inhibit recruitment of MO-MDSC.

Conversely, the Matthew J. During group has shown that an enriched environment (EE) inhibits tumor progression in a mouse model [95]. Practically, C57/BL6 mice were housed in large cages of 1.5 m $\times$ 1.5 m $\times$ 1.0 m with 18–20 mice each, which were equipped with running wheels, wood toys, etc., while control mice were in standard cages. They claimed that luxurious space, physical activity, and social interactions were achieved by the EE in those mice whose serum suppressed cell growth of B16 melanoma cell and MC38 colon cancer cells *in vitro*. Mechanistically, brain-derived neurotrophic factor (BDNF) from hypothalamus, inhibited leptin expression in white adipose tissues (WAT), which led to reduced angiogenesis and suppression of tumor growth in the EE mice. BDNF-KO mice displayed an inhibitory influence on the *in vivo* EE phenotypes.

It is interesting to consider that BDNF can be induced by 5-HT, whose deficiency is a well known sign of depression. Although the precise mechanisms are still unknown, it is clear that three parts, CNS, WAT, and tumor, are interconnected, and CNS is controlled by environmental factors.

12.7.2.4 Self-Seeding Hypothesis

Tumor cells that extravasate into circulation from the primary site come back and intravasate into the primary site after stopping by other organs or tissues in the body for activation or “education.” In this case, interplay is supposed to be between two or more time points of the same primary site, an old primary site before self-seeding

and a new primary site after it. Experiments can be designed by injecting unlabeled and GFP-labeled breast cancer cells into right and left mammary gland, respectively, with the former serving as a recipient tumor mass on the right that receives GFP⁺ cells derived from the GFP⁺ tumor mass on the left. Tumor-secreted IL-6 and IL-8 appeared to be responsible for attracting self-seeding tumor cells [96]. CXCL1-knockdown in tumor cells resulted in reduction in the number of recruited CD45⁺, mainly CD11b⁺ myeloid, cells in the seeded tumor responsible for elevated tumor growth and angiogenesis. The similar phenomenon was observed with colon cancer cells and melanoma cells.

12.7.3 Lymphatic Spread

Because lymphatic ducts eventually drain into systemic circulation (Fig. 12.2), tumor cells that make an entry into lymphatic ducts with weaker barrier structures within the primary tumor (see Chap. 11) also can achieve hematogenous spread as exemplified in lung metastasis. However, spreading to either regional or other lymph nodes seems to be determined by an as yet unknown mechanism. Intratumoral lymphatic ducts are usually collapsed by physical pressure of the solid tumor. VEGF-C as well as VEGF-D is secreted by a variety of tumor cells and stromal macrophages. VEGF-C binds VEGFR2, VEGFR3, and the coreceptor NP-2, which can enhance the VEGFR signaling.

Anti-VEGFR3 blocking antibody could reduce intra-tumoral CD31-monitored angiogenesis and LYVE-1-monitored lymphangiogenesis in human NCI-H460-LNM35 lung cancer xenograft in SCID mice but not preexisting dermal lymphatic vessels that overlie the tumor [97]. Administration of adenovirus expressing a human soluble VEGFR3 decoy receptor via portal vein sustained the sVEGFR3-Fc levels for more than 4 months [98]. This virus could reduce not only lymphangiogenesis within the subcutaneously implanted tumor but also lymph node metastasis in renal cancer Caki-2 and prostate cancer PC-3 nude mice models.

Establishment of anti-NP-2 antibody could dissect the lung metastasis derived from lymphatic and hematogenous spread [99]. It bound NP-2 with K_d around 5 nM and inhibited phosphorylation of VEGFR 2 and VEGFR3 and suppressed VEGF-C-stimulated cell migration in lymphatic endothelial cells. The antibody failed to inhibit growth and CD31-monitored tumor angiogenesis in the subcutaneously injected primary tumor of either NP-2-expressing 66c14 mammary carcinoma cells or NP-2-nonexpressing C6 tumor cells but abrogated their metastatic progression to the lungs by roughly 40 % as judged by micro-CT-mediated metastatic tumor volumes. In the unaffected primary tumor, LYVE-1-monitored lymphangiogenesis was impaired. Those experiments clearly indicate that anti-NP-2 inhibitory antibody could inhibit lung metastasis not by directly targeting tumor cells by themselves but lymphangiogenesis within the primary tumor.

12.8 Application of the Triangle Idea to Tumor Cells (See Fig. 3.6 in Chap. 3)

The trigger of triangle activation is tumor cells or factors produced from the tumor.

12.8.1 Tumor Cells Activate Coagulation

Coagulation activity in tumor tissues was originally described by RAQ O'Meara and RD Jackson in 1958 [100]. They not only noticed deposits of unresolved fibrin, the end product of coagulation, in fresh biopsy materials from a wide range of carcinomas by careful cytological observation, but also performed in vitro experiments to show the presence of coagulation factors in the malignant tissues compared with apparently normal tissues of the specimens from which the malignant tissues were derived. Detailed pathological analysis of lung cancers revealed that colocalization of III and VEGF as well as fibrin deposits predominantly on the surface of TAM.

In many tumor tissues, III expression is up-regulated. VEGF and TNF α derived from both tumor cells and macrophages stimulate endothelial cells to produce III, although this was reported to be counteracted by the Ang1-Tie2 pathway. Binding of III expressed on the cell surface of human lung fibroblasts to VIIa resulted in VEGF production.

Thrombin-stimulated PAR1 elicits Ca²⁺ signaling that releases ADAM10 from calmodulin. The translocated ADAM10 to the plasma membrane cleaves VE-cadherin to induce vascular permeability.

In electron microscopic analysis of tumor emboli in rat lungs after intravenous injection of Walker 256 tumor cells through the tail vein, fibrin appeared by 5 min in the platelet clumps associated with tumor cells, peaked around 60 min and disappeared at 9 h. This old experiment from 1976 clearly shows that tumor cells not only at the primary site but also in other parts, as those at the distant metastatic sites, of the body can activate the coagulation system [101].

Many papers have documented the relationship between coagulation and cancer in clinical settings. For example, analysis of 69 samples of clear cell renal cell carcinoma that is closely associated with VHL deficiency-induced VEGF overexpression revealed that high VEGF expression correlated well with high fibrin staining [102].

The biological significance of platelet has been known for a long time as represented by classical experiments by the Takashi Tsuruo group in 1997, showing that antiplatelet antibody was capable of inhibiting lung metastasis 14 days after tail vein injection of highly metastatic mouse colon cancer cells NL-17 [103].

Cytoplasmic concentration of Ca²⁺ in platelets increases when activated by collagen or thrombin. Sarcoplasmic reticulum (SR) is the major storage source, Ca²⁺ deprivation of which induces a sustained influx of extracellular Ca²⁺. The

sensing mechanisms involve stromal interaction molecule 1 (STIM1) and Orail 1, also called CRACM1, was identified as the channel for the store-operated Ca^{2+} entry (SOCE) [104]. Orail1-KO mice displayed resistance to arterial thrombosis [104]. The second messenger Ca^{2+} participates in a variety of cell types, such as platelets, fibroblast, and tumor cells. A channel blocker SKF96365 could abrogate cell migration of human breast cancer cells MDA-MB-231 and their lung metastasis in xenograft experiments in NOD/SCID mice. The authors documented that the drug that was intraperitoneally administered for 4 weeks from the time of tumor cell injection through the tail vein remained biologically effective to suppress metastatic progression in the lungs during the subsequent 2 weeks after drug withdrawal. The drug efficacy also was confirmed by lung metastasis model of 4T1 mouse mammary tumor cells, although they did not show any information on coagulation.

SOCE-mediated oscillation of intracellular Ca^{2+} concentration as monitored by a chemical Ca^{2+} indicator Fluo4-AM, which interacts only with freely diffusible Ca^{2+} , in WM793 human melanoma cells can be induced by serum and blocked by SOCE inhibitors, such as SKF96365 and 2-APB but not an L-type voltage-gated Ca^{2+} channel inhibitor nifedipine [105]. Chelating extracellular Ca^{2+} by EGTA significantly reduced the frequency of the oscillation from 18.6 to 3.3/h. The oscillation correlated with formation of invadopodia that manifests actin-rich membrane protrusion degrading ECM by recruiting MT1-MMP. Tail vein injection of a highly metastatic variant of WM793 cells in nude mice induced lung metastasis in 36 days, which could be reduced by more than 80 % by anti-STIM1 shRNAs. The same authors showed STIM1 and Orail were overexpressed in many melanoma cells compared with normal melanocytes. Interestingly, although SOCE regulated invadopodium via Src and constitutive Ca^{2+} influx by thapsigargin also activated Src, thapsigargin inhibited invadopodia formation.

We also should remember the old experiments by Takashi Tsuruo of the platelet aggregation-inducing activity associated with tumor cells, blockade of which by neutralizing antibody suppressed lung metastasis [106, 107]. The antigen turned out to be identical to podoplanin, which was initially thought to be rather specifically expressed in lymphatic endothelial cells (see Chap. 11). However, podoplanin is now known to be expressed in a variety of cells, including kidney podocytes and alveolar type I cells [108]. The only known ligand CLEC-2, a C-type lectin, is expressed in platelets and binding to podoplanin is mediated by O-linked glycosylation in podoplanin. Aberrant podoplanin expression in tumor cells is under the control of AP-1, which is activated by various stimuli in tumor microenvironment, and podoplanin signaling is assumed to play a role in tumor invasion.

Clot that surrounds tumor cells could limit access of NK cells [109]. In fact, pulmonary metastasis is reduced in the background of fibrinogen or fibronectin knockout.

12.8.2 Tumor Cells Activate Angiogenesis

12.8.2.1 VEGF

Tumor tissues secrete high levels of VEGF in a manner dependent on both growth-based AP-1 and hypoxia-based HIF-1. Oxygen pressure is sensed by PHD as stated in Chap. 11. In PHD2+/- mice with poor sensing ability of oxygen, intravasation of tumor cells and subsequent metastatic progression were inhibited, which is accompanied by increased expression of VE-cadherin and sVEGFR1. Many tumors have an increased expression of SphK1 responsible of catalytic conversion of sphingosine (S) to sphingosine 1-phosphate (S1P) (Fig. 3.4 in Chap. 3). Activation of S1P receptor 1 promotes expression of both VEGF and PDGF.

Antiangiogenesis therapy against cancer was proposed by Judah Folkman more than 40 years ago and was tested in clinical settings by anti-VEGF antibody and VEGFR tyrosine kinase inhibitors [110]. However, it has had less effective results than expected. Discussion on this issue would provide us with several ways to consider cancer in the triangle.

For example, comparative pathological analysis of recurrent glioblastoma autopsy samples derived from cediranib-treated five patients and seven patients who were treated by nonantiangiogenic agents, including chemoradiation, showed decreased cell density in the central region but not infiltrative edges. Although they found no signs of revascularization after discontinuation of the cediranib treatment, both tumor cells in the center and infiltrating cells expressed c-Met [111]. The HGF-c-Met signaling not only promotes tumor cell growth but also ensures angiogenesis.

Pretreatment of SCID mice with high doses of sunitinib, a VEGFR inhibitor, daily for 7 days before injection of 231/LM2⁻4luc⁺ tumor cells via the tail vein significantly accelerated the metastasis [112]. Also in an orthotopic xenograft model with the same tumor cells, surgical resection induced spontaneous metastasis in the lungs and liver, which was aggravated by sunitinib treatment.

However, in contrast to the prometastatic effects of sunitinib observed in murine studies, a recent report on the final results of the large sunitinib global expanded-access trial in metastatic renal cell carcinoma (N = 4,543, metastasis in lungs 76 %, bone 35 %, liver 27 %, and brain 7 %) with 4,219 evaluable patients revealed clinical benefits (objective response and stable disease for more than 3 months) in 61 % and those for brain metastasis group in 42 % [113].

Although mechanisms are still unclear, propranolol, a β -blocker, is currently utilized for effective treatment of hemangioma [114]. Propranolol has been shown to potentiate antiangiogenic effect and cooperative effects with antineoplastics, such as paclitaxel.

12.8.2.2 Hypoxia

Although inhibitors against the growth-promoting EGFR-Ras-PI3K-Akt pathway could lighten the hypoxic burden of tumor cells, anti-VEGF antibody could not only inhibit active tumor angiogenesis but also destabilize vascular barrier leading to activation of vascular permeability and coagulation system. This results in aggravation of hypoxic condition in tumor cells, promoting HIF-1-dependent up-regulation of SDF-1 (CXCL12), CXCR4, PIGF, HGFR alternatively called c-Met, Lysyl oxidase (LOX), G-CSF, and IL-6. Thus, exacerbated hypoxia causes inflammation-producing growth factors, such as FGF, instead of ameliorating VEGF-mediated inflammation [115].

SDF-1 (CXCL12) stimulates its own receptor CXCR4 to induce HO-1 in a PKC ζ -dependent manner [116]. SDF-1 can induce sprouting in aortic ring assays in a HO-1-dependent manner, which can be rescued by CO, the byproduct of HO-1. Although the precise mechanisms need to be uncovered, the potential role of CO in the resolution phase is discussed in Chap. 11.

SDF-1 stimulates cell migration of human microvascular endothelial cells at 100 ng/ml, which was equivalent to that induced by VEGF at 20 ng/ml by one report, tube formation in vitro in a fibrin gel and neovascularization in rat corneal in vivo angiogenesis assay. In mice, it is thought that CXCL12-CXCR4 signaling induces mobilization of bone-marrow-derived endothelial progenitor cells in circulation, which participate in vessel formation. However, it is controversial in humans and evidence is missing, as far as I am aware, about the development of restricted endothelial lineage of cells to form lumen in stabilized vessels when they are transplanted [70].

12.8.2.3 PIGF-VEGFR1-Mediated BMDC Migration

Transcriptome analysis of endothelial cells (EC) revealed overexpressed genes preferentially in tumor, such as CT26 metastatic colon cancer cells compared with normal vessels, including PIGF [117]. Mobilization of bone marrow-derived cells (BMDC) not only to primary tumors but also potentially metastatic sites is mediated by SDF-1 and PIGF. PIGF is a specific agonist for VEGFR1 expressed in BMDC particularly in monocytes. A cDNA microarray analysis of endothelial cells between physiological and pathological angiogenesis revealed PIGF. The authors showed that anti-PIGF antibody could inhibit pulmonary metastasis with neither affecting the size of primary tumors nor tumor angiogenesis. BMT with bone marrow cells in which VEGFR1 was knockdown by lentivirus-mediated anti-VEGFR1 shRNA followed by B16 melanoma cell transplantation gave significant reduction in lung metastasis.

Moreover, PIGF could directly promote tumor cell growth dependently on neuropilin-1 (NP-1) and independently of VEGFR1 in a human medulloblastoma xenograft model [118]. A triggering mechanism is secretion of human PIGF from

the tumor, which induces not only the tumor growth via NP-1 but also bone marrow cell mobilization to the tumor. The tumor cells also secrete shh ligands to stimulate stromal cells to secrete mouse PlGF, further stimulating the tumor cells for survival through NP-1 but not VEGFR1. Interestingly, they claimed that anti-NP-1 antibody inhibited the tumor growth and anti-PlGF antibody had no effect on vascular permeability and TAM recruitment.

12.8.2.4 G-CSF

G-CSF induces mobilization of BMDC and stimulates them to secrete Bv8 capable of causing angiogenesis in a VEGF-independent manner [119, 120]. This is indeed one of the mechanisms of resistance against anti-VEGF therapy. The source of G-CSF includes tumor cells transformed by the Ras-MEK pathway [121] and host CAF through NF κ B and Erk signaling. Resistance to anti-VEGF therapy could be overcome in IL-17R-KO (precisely, IL-17A binds a heterodimer of IL-17RA and IL-17RC, and here IL-17R-KO means KO of IL-17RC) or G-CSFR-KO. IL-17A from tumor-infiltrating Th17 cells induced G-CSF in CAF [122]. Up-regulation of G-CSFR in the hyperpermeability regions in the premetastatic lungs (see Chap. 13) of tumor-bearing mice also may serve as good soil for G-CSF-producing tumor cells to achieve metastatic growth [123].

12.8.2.5 Pericyte Damage

Stabilization of vasculature requires pericytes. Sunitinib not only inhibits VEGFR2 to suppress endothelial proliferation but also PDGFR to destroy vascular barrier due to pericyte damage [119].

The above-mentioned events collectively give resistance to antiangiogenesis therapy. Tumor cells become invasive under VEGF inhibition but remain invasive after stopping VEGF inhibition. Aggravated hypoxia induces production of ROS, which not only inactivates O₂ sensor PHD2 but also induces DNA damages and subsequent mutations.

12.8.2.6 Angiotropism Induction

Plasmin converted from plasminogen by plasmin activator (PA) is engaged in fibrinolysis. Given that clot formation favors tumor progression, plasmin is likely to be antitumorigenic. However, it depends on the biological context. Plasminogen-KO had no effect on lung metastasis of LLC that were injected through the tail vein. An opposite activity also was reported. Plasmin proteolytically activated the transmembrane protein CUB domain-containing protein-1 (CDCP1), which in turn elicited survival signaling through Src and Akt for tumor cells that were colonized in the lungs [124].

PA is homeostatically controlled by serpin family of proteins (see Chap. 11), such as PA inhibitor-1 (PAI-1, also called serpin E1). Again the effect of PAI-1 seems to be dependent on the context. While brain metastasis by retinal pigment ocular tumors in tyrosinase-related protein 1/SV40 T antigen transgenic mice was suppressed in PAI-KO [125], a cohort of 63 transgenic mice of MMTV-PyT murine mammary tumor model under the background of PAI-1-KO failed to affect lung metastasis [126] (see also Chap. 16). More recently, transcriptome signature analysis of brain metastatic tumor cells revealed that the tumor cells expressed other serpins, such as serpin B2, and neuroserpin, which is physiologically produced by neurons to counteract against PA that is expressed in injury-responsive astrocytes. Those serpins were found to be expressed in 34–94 % of clinical samples of brain metastasis in breast and non-small lung cancer [127]. In this case, plasmin also was claimed to be responsible for L1CAM cleavage that caused tumor cell death. Collectively, homeostatic controls of coagulation additionally influence tumor cell survival by cleaving membrane proteins that are involved in cell survival in a context-dependent manner.

12.8.3 Tumor Cells Activate Inflammation

I have described the details of tumor cell-associated inflammation in 12.3 through 12.7 under this heading.

12.9 Concluding Remarks

Both tumor cells and immune cells come in and out of not only the primary tumor tissue but also other nonimmune organs, such as lungs. Placing tumor cells in the triangle center of coagulation, angiogenesis, and inflammation by utilizing a variety of mouse metastasis models may help to understand the complicated molecular events played by those tissues as a matter of whole-body.

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Part IV
Homeostatic Inflammation And Metastasis

Chapter 13

Premetastatic Microenvironment

13.1 Overview

I believe that the inflammation within the primary tumor that intrinsically progresses over time and can be augmented by any procedures, physical or chemical, aggravates the metastatic progression of the primary tumor unless the procedures can impair the tumor cell survival at least partly. For example, skilled surgeons can remove a tumor with minimal tissue damage as represented by little blood loss during operation. Earlier removal of the primary tumor is better for cure, because it might be before the regrowth of the metastasizing tumor cells. Once they begin regrowth, the situation is similar to that in the primary tumor.

One of the excellent reviews on metastasis as exemplified by the Joan Massague group focuses on acquisition of metastatic trait by tumor cells, meaning that tumors are basically heterogeneous and subjected to selection during the process of metastasis by extra-tumor microenvironment, such as circulation and metastatic tissues [1]. I'll start by returning to the old, but still real, idea of seed and soil hypothesis by Stephen Paget and describe the relationship between primary and metastatic site.

13.2 Clinical Information

13.2.1 *Stephen Paget 1889 (see Table 9.1 in Chap. 9)*

Prediction of distant organ microenvironment (soil) cultivated by the primary tumor consisting of tumor cells (seeds) and stromal cells was made with careful analyses of statistical significance of necropsies by Stephen Paget in 1889. Evidence was the disproportionate distribution of metastatic organs in cancer compared with sepsis with metastatic abscess called pyemia. In 735 cases of breast cancer, 244 cases of uterine cancer, and 340 cases of pyemia, the ratios of metastatic involvement in

liver and spleen were 14:1 in breast cancer, 35:1 in uterine cancer, and 1.7:1.0 in pyemia [2]. It was quite reasonable for him to come up with the idea that the circulation-based phenomenon was not due to chance.

13.2.2 Metastatic Features without Primary Tumors

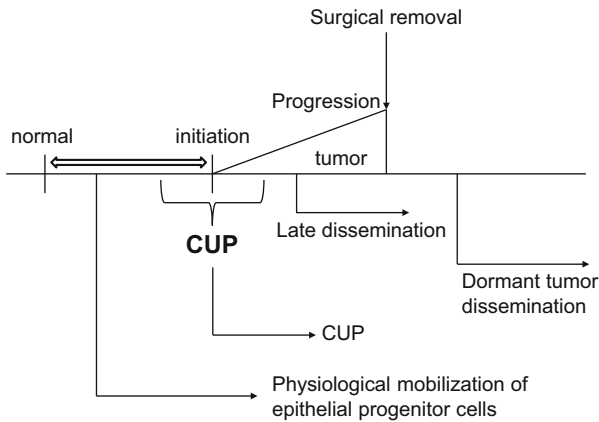
I suppose that there are three principal stimuli responsible for triggering inflammation: (1) infection, (2) injury, and (3) noninjurious and infection-mimicking. The last two stimuli cause noninfectious or sterile inflammation. In this section, I try to prove that premetastatic microenvironment is devoid of microbes and tissue injury but maintains inflammatory features by the continuous remote control of a primary tumor at a distant site.

13.2.2.1 Cancer of Unknown Primary

Metastatic sites never exist without corresponding primary tumor. This is generally true. However, clinical evidence certainly shows the existence of cancer of unknown primary (CUP), also called occult carcinoma or tumor-orphan metastasis [3] (Fig. 13.1). The incidence ranges between 0.5 % and 7.0 % of tumor registry [4]. An alleged notion is that the corresponding primary tumor is too small to be detected by the current level of the accuracy of imaging study [3, 5]. The word <too small> means either an existence of a small cluster of primary tumor cells with highly metastatic traits but under clinical detection or an absence after spontaneous tumor regression. CUP clearly shows that metastasis can take place irrespective of the original tumor size.

In a report in 1986, 1,539 cases of CUP, which was defined by the criteria that the origin could not be uncovered within 4 months of diagnosis, constituted 3 % of

Fig. 13.1 Cancer of unknown primary



total registered tumor patients. Confirmed diagnosis by microscopy in 1268 cases showed that the most frequent one was adenocarcinoma (38.2 %) and a variety of cancers were included, such as melanoma (2.9 %) [4]. An intriguing observation was made on CUP with defined primary sites at autopsy that pattern of metastatic sites in CUP (for example, lung and liver in CUP of autopsy-defined prostate cancer) is rather different from that in known primary tumors (usually bone in clinically-diagnosed prostatic cancer) [3]. However, recent trials to identify CUP molecular signatures by gene expression profiling, including EGFR, p53, and MMPs, have so far given elusive results [6].

13.2.2.2 Effects of Primary Tumor Resection

It is no wonder that clinical influences on the prognosis and future metastasis after resection of the primary tumor have been either beneficial or harmful, because we can neither determine the accurate stage of progression of each tumor of various origins at the time of operation, nor normalize the surgical skills and techniques.

Direct evidence to support the idea that CTC persists after removal of the primary tumor has been provided. Low concentration of steady breast cancer CTC, CD45⁺CK⁺ cells, was detected 7–22 years after mastectomy in 36 % of patients even in the absence of the disease compared with 3.8 % in an age-matched control. The short half-life of 1.0–2.4 h in CTC strongly suggests the dormant breast cancer cells somewhere in the body releasing CTC that eventually undergoes apoptosis or elimination in the reticuloendothelial system [7].

A precious report of lung metastasectomy in already nephrectomized renal cell cancer patients has been made and, combined with the information in the literature, they suggested some benefits [8]. Collectively, clinical evidence suggests that metastasis takes place even at the very early stages of tumor development when the primary tumor is large enough to be visually detected as observed in CUP or after successful removal of the primary tumor.

13.3 Experimental Information

In a mouse tumor model of subcutaneous implantation, Ernest Edward Tyzzer showed in 1913 that physical damage by massage of the primary tumor in the premetastatic period, which he referred to as the time before recognition of lung metastasis by histological examinations after inoculation, and partial removal of the primary tumor facilitated the growth of metastatic nodules [9]. In a rabbit tumor model, a chemical injury of spleen by injection of nitrogen mustard into gastric branch of splenic artery followed by intra-aortic inoculation of the tumor cells also promoted splenic metastasis. The effects of primary tumor resection on the subsequent metastatic progression in experimental animals have been similar to those in the clinical settings being either inhibitory or stimulatory.

Alfred S. Ketcham showed in 1961 by detailed analyses of five different systems that removal of the primary tumor decreased the number of metastatic foci in the lungs, but the size of the foci increased, including Cloudman S91 melanoma in CDBA/2F1 mice, melanoma S91A in DBA/p, osteogenic sarcoma 112 in C3HfB/HeN, T241 Lewis sarcoma in C57BL/6JN, and DBA49 in DBA/p [10]. The information provides the general basis on which surgical removal of the tumor should be done in clinical setting and suggests that the persistent presence of the primary tumor releases CTC that reaches the lungs, which is suppressed by its removal. The status of the disseminated tumor cells, whether dead, dormant, initiating regrowth, or already regrowing, for potential lung foci in the future differs, because they reach there at different time points.

The Isaiah J. Fidler group in 1997 documented that C57BL/6 mice subcutaneously implanted metastatic 3LL tumor cells gave rise to a tumor with spontaneous lung metastatic foci whose mean number was 10, which was drastically increased to 61 with bigger sizes when the tumor was resected [11].

There has been an interesting phenomenon called <concomitant tumor resistance> in which tumor-bearing hosts are protected against the growth of second tumor challenge by implantation or forced metastasis by direct injection through vessels [12]. Removal of one C3H mammary tumor from the doubly implanted C3HeB/FeJ mice induced an increase in DNA synthesis in the other tumor within 24 h after removal [13].

Fractionation of serum from mice bearing LB tumor, a spontaneously occurring T-cell lymphoma in BALB/c, revealed an inhibitory fraction containing meta-tyrosine and ortho-tyrosine for in vitro growth of LB tumor cells. The paradox that those tyrosine isoforms produced from the primary tumor of larger size are inhibitory to the secondary implant of smaller size but not to the primary one is explained by the more abundant presence of counteracting amino acids, i.e., Phe, Glu, Asp, Gln, and His in the primary than the secondary. Those tyrosine isomers are shown to suppress both MAPK and STAT3 [14].

Another antagonistic double control is VEGF and TSP-1 (see Chap. 11). Human fibrosarcoma HT1080 transplanted in nude mice confers resistance to lung metastasis by intravenously injected B16/F10 melanoma cells. The resistance was shown to be caused by the tumor production of TSP-1 whose knockdown by antisense strategy abrogated the resistance. The plasma concentration of the secreted TSP-1 was approximately 6 µg/ml high enough to exert systemic antiangiogenic effects as monitored by mouse cornea assays. The plasma half-life of VEGF is 30 min and that of TSP-1 is 9 h [15–17]. In the primary tumor, the paracrine effect of VEGF is likely stronger than TSP-1. In the distant lungs, TSP-1 with a longer half-life appears to overcome the VEGF effect. Removal of the tumor producing both of them would release the TSP-1-mediated suppression. If the primary tumor produces an inhibitory substance for the regrowth at metastatic sites, the deinhibition by its removal would result in enlargement of the metastatic foci. This idea has been supported by the angiogenic switch model proposed by Judah Folkman (Fig. 13.2). In fact, the patient from whom HT1080 is derived died of disseminated tumor 3 months after removal of the primary tumor [18].

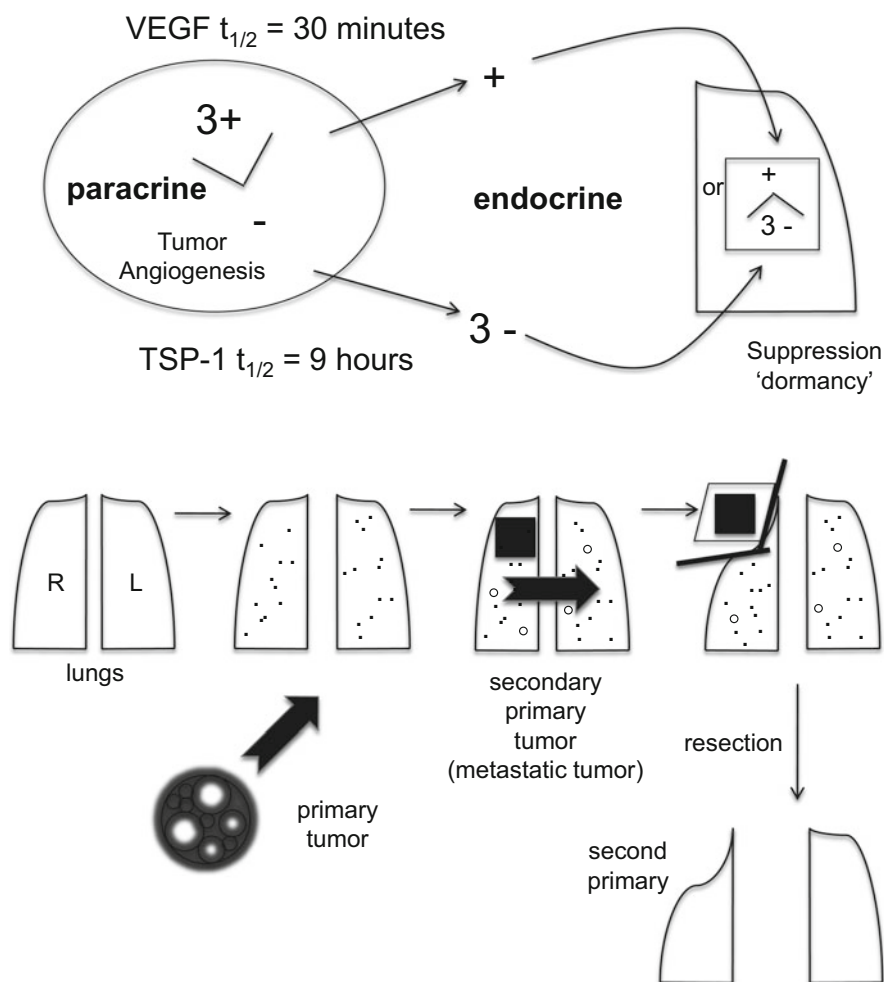


Fig. 13.2 Angiogenic switch model. Balance of positive and negative factors in metastatic distant tissues is influenced by their activity and bioavailability as exemplified by the half-life in circulation

Positive regulators in angiogenic switch are VEGF, bFGF, and PDGF, and negative ones are PF4 and TSP-1. The inhibitory mechanisms in angiogenesis are stated in Chap. 11. Importantly, antagonistic controls in angiogenesis innervate distant tissues in an endocrine fashion. All of those molecules are embedded in platelets with concentrations of VEGF of 0.74 pg, bFGF 0.44 pg, TSP-1 31 ng, and PF4 12 ng per 10^6 platelets [19, 20]. The concentrations of the inhibitory molecules are roughly 4 magnitude higher than proangiogenic ones. I presented the triangle of inflammation—angiogenesis—coagulation in Chap. 3. If one of the three can

metastasize from the first implanted tumor to the doubly implanted second tumor or other normal tissues in the body, the triangle also could metastasize (see below).

13.4 Concept of Field

Field carcinogenesis helps us to understand “field metastasis” and premetastatic soil (microenvironment) before stochastic arrival and regrowth of metastatic tumor cells.

13.4.1 *Within a Single Organ*

I will explain field carcinogenesis in colorectal cancer. The whole colon as an organ is recognized as a field where time-dependent alterations proceed diffusely throughout the entire colorectal mucosa. The sites of cancer development are determined during the course of the field changes stochastically by for example superimposed mutations of Ras on APC. For example, if you find something in the rectal mucosa, which is clinically convenient to test, you can predict development of colon cancer in the ascending colon without directly performing extensive colonoscopy. Reports on both cDNA microarray and proteomic analyses revealed alterations in the expression levels of genes, such as TGF α and epigenetic IGF2, in the seemingly normal rectosigmoid mucosa derived from patients with proximal adenocarcinoma or adenoma [21–23]. Needless to say, an ideal noninvasive way to predict the presence of cancer is by just looking at a part of the colorectal field, such as rectal mucosa. One such clinical method is to detect an early increase of microvascular blood supply (EIBS) by polarization-gated optical spectroscopy. Generally, colorectal cancer is more dependent on angiogenesis for growth than other cancers, such as breast cancer. If the field is extended to whole colon + blood, elevations of serum concentration of CEA, one of the biomarkers of colorectal cancer, can serve another change of the field colon carcinogenesis (Fig. 13.3).

Taking this idea into premetastasis, premetastatic lungs form field metastasis. The diffuse recruitment of CD11b-positive cells in the whole lungs with some preference to hyperpermeable regions that develop in a segmental manner is the alteration of the field (see below). Seeding of metastatic tumor cells as well as regrowth stochastically determine the spatial position of metastasis.

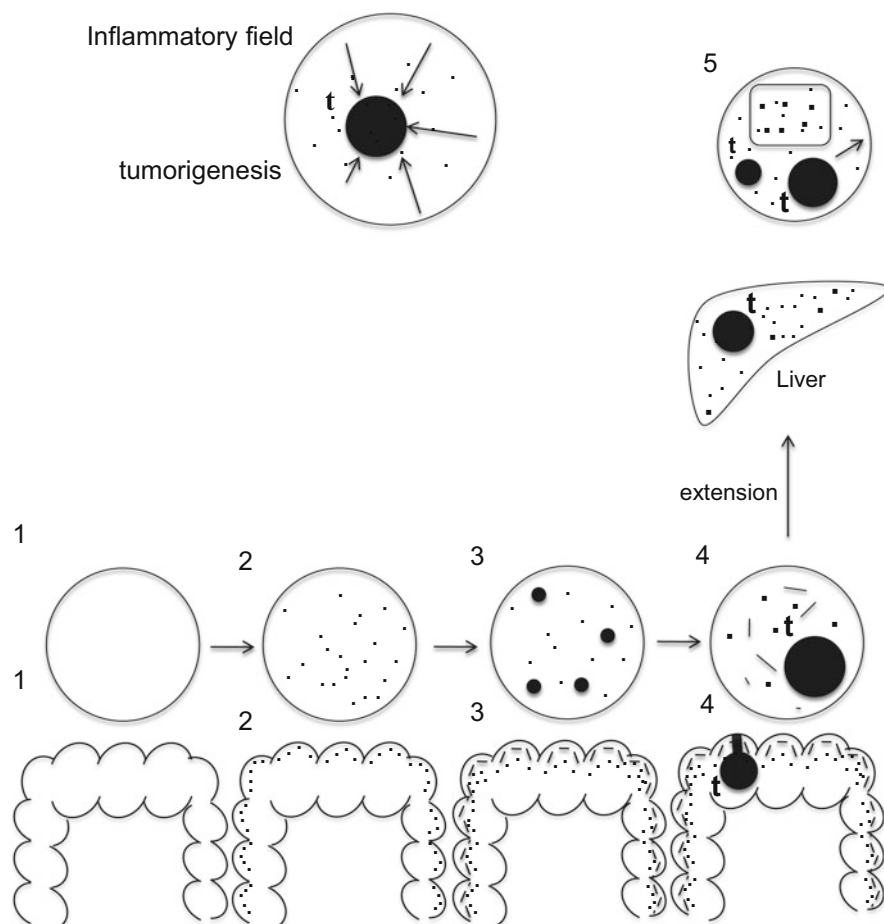


Fig. 13.3 Field extension. Tumor-developing microenvironment may be recognized in any parts of the soil, the whole colon in this figure, in which a tumor develops in a stochastic fashion

13.4.2 Primary Inflammatory Lesion can “Metastasize” to Other Organs

Most locally generated inflammation eventually subsides. However, local inflammation can metastasize to other parts of the body. Evidence can hardly be drawn to prove that those more than two inflammatory sites are based on a single cause. Here I will discuss extraintestinal Crohn’s disease. Although mechanisms of the disease remain uncovered, approximately 40 % of patients are thought to involve skin ulceration or anal fistula or abscess in any time point of the clinical course. Ulcerative lesions in the affected intestine are demarcated and segmental with intact intestine in between Deep, transmural, and penetrating ulcers in the anus

may initiate fistula formation distant from the lesions in colon. Systemic administration of anti-TNF α antibody, such as infliximab and adalimumab, has shown significant efficacy [24]. One of the key pathological hallmarks of Crohn's disease is the chronic granulomatous reaction with noncaseating multinucleated giant cells in epithelioid and lymphocytic cells, which is found in ulcerative lesion and regional lymph nodes. Presence of those pathological characteristics in skin lesions guarantees that both ulcers are of the same disease, and it often is said that the primary intestinal lesion metastasizes to the skin by analogy to tumor metastasis [25]. However, this is not just an analogy in a literal sense, because I believe and explain in this chapter that tumor metastasis is based on "metastasis" of inflammation, i.e., transfer of inflammation of one organ to another (inflammatory metastasis precedes that of tumor).

Inflammation seemingly spreads from site A to B, but it is mediated by bone marrow. Although blood cells may infiltrate into B from A, it is thought that blood cells originate from bone marrow for infiltration into both A and B sites. In tumor metastasis, myeloid cells disseminate into both the primary and premetastatic sites from bone marrow. It holds true even in the absence of tumor cells as exemplified by Langerhans cell histiocytosis (LCH), alternatively called histiocytosis X (HX) from a long time ago [26]. The affected organ is either single or multiple with inflammatory lesions, including langerin+ DC, macrophages, NK cells, T and B cells with increased tissue expression of CCL2, IL-6, IFN γ , and TGF β . BRAFV600E mutation is found in 64% of the HX patients. While conditional mice that express BRAFV600E under the langerin promoter, which is active only on differentiated tissue resident DC but not their progenitor, displayed only minor lesions in liver, those under the CD11c promoter exhibited systemic HX-like phenotypes [27]. This shows that alterations of progenitors in bone marrow could take place to provide multiple inflammatory lesions in the body.

13.5 Definition of Premetastasis

Premetastatic microenvironment is a host-side tissue condition prepared by remote controls of distant tumor before the actual arrival of disseminating tumor cells from the primary site. The idea is interesting, but the question is how to prove the existence.

13.5.1 *Forced Experimental Metastasis*

Before we see the experiments of premetastasis, I present two experiments to show that forced metastasis could be successful but with low efficiency. Old experimental results exist to show that the tumor-controlled preparation is not necessarily required for metastasis to take place. For example, in 1970 Isaiah J. Fidler labeled

B16 mouse melanoma cells with ^{125}I -iodo-2'-deoxyuridine, injected them into the tail vein of syngeneic C57BL/6J mice and quantitatively followed the number of tumor cells in the lungs, liver, and blood as determined by the radioactivity, up to 14 days when the mice were subjected to pathological examinations [28]. The tumor cell numbers increased up to 1 h after injection mostly in the lungs and liver, but the liver count showed less than one tenth of that of the lungs. Approximately 1 % and only 0.004 % (i.e., 78 lung metastatic foci out of 2×10^5 injected cells) of the injected tumor cells survived in the lungs at 24 h and formed metastatic colonies in the lungs at 14 days, respectively. No liver metastasis was detected.

In 1998 with the development of experimental procedures, the Alan C. Groom group labeled B16F1 melanoma cells using fluorescent nanospheres, injected them into the portal vein of the syngeneic mice, and observed the spread tumor cells in the liver by utilizing in vivo videomicroscopy and immune-staining, such as Ki-67 to monitor their proliferation [29]. Approximately 80% of the injected cells accomplished extravasation; only 2 % of cells started proliferation to form micrometastasis consisting of 4–16 cells by day 3. Macrometastatic foci were found in 0.018 % by day 13. Importantly, they observed solitary tumor cells that achieved extravasation and 95 % of them stayed dormant, i.e., negative in both TUNEL (apoptosis) and Ki-67 (proliferation). Under the same methods, they examined the tumor cells in micrometastasis and found that 3.3 % were of dormancy.

With those two experiments, we can now understand what I stated at the beginning. I underline that the solitary survival of extravasated tumor cells under detection in clinical settings could potentially give rise to metastatic growth.

13.5.2 Premetastasis Experiments

Evidence that the David Lyden group showed in 2005 was (1) a negative study in DsRed-labeled B16 melanoma cells in the lung preparation by flow cytometry earlier than day 16 after tumor cell implantation in the subcutaneous tissue, and (2) serum factors produced by the B16 tumor cells in vitro mobilized GFP-positive cells in mice that had undergone bone marrow transplantation by EGFP transgenic mice [30]. This pretreatment enhanced lung recruitment of the intravenously injected tumor cells. The above-described evidence gave us an assumption that tumor cell-derived factors may alter the pulmonary microenvironment before the tumor arrival.

Tumor cells cultured in vitro are basically different from the primary tumor in which the tumor cells coexist with a variety of surrounding or stromal cells. Solid evidence should be (1) to show cutoff values to exclude the presence of any single tumor cells in the lungs by any methods, (2) to identify the serum factors produced by tumor cells or the primary tumor responsible for recruitment of both bone marrow-derived cells (BMDC) and tumor cells to the lungs, and (3) to show that BMDC is necessary and sufficient for the tumor cells to extravasate to the lungs.

The original paper lacked the evidence (1) and (2) but supported (3). Expression of fibronectin was increased 3 days after LLC implantation up to day 14, to which VEGFR1⁺ myeloid cells were recruited through the fibronectin receptor VLA-4 and depletion of VEGFR1⁺ BMDC from the recipient mice by anti-VEGFR1 antibody impaired recruitment of both BMDC and the B16 tumor cells. Transplantation of purified VEGFR1⁺ BMDC enhanced metastasis.

In what they call premetastatic microenvironment with VEGFR1⁺ myeloid cells, fibronectin and fibroblasts were gathered together, and CXCL12 expression was increased. They explained that the presumed CXCL12 gradient may attract B16 or LLC cells expressing its receptor CXCR4.

However, I need to introduce one experiment by the Rakesh K. Jain group with detailed analysis of BMDC and an opposite conclusion on VEGFR1. They subcutaneously implanted B16 or LLC tumor cells, both of which lack VEGFR1 expression, to the mice whose bone marrow had been reconstituted that from GFP mice to monitor BMDC by GFP images. The size of the primary tumor grown in the premetastatic period of 14 days after implantation was affected neither by anti-VEGF antibody nor by the recipient background of TK^{-/-} mice in which the tyrosine kinase domain of VEGFR1 had been deleted. The number of BMDC within the tumor and premetastatic lungs were not influenced by anti-VEGFR1 antibody. At day 14, the primary tumor is removed to facilitate spontaneous lung metastasis, which became macroscopically prominent in the subsequent 14 days. Anti-VEGFR1 antibody could reduce the number of BMDC in the lung metastatic nodules formed by LLC but not B16 [31].

We showed in 2006 through 2013 that, by defining the cutoff value as less than 100 tumor cells per lung lobe as judged by PCR analysis of the neomycin-resistant gene that had been retrovirally integrated into the genome of the injected tumor cells, serum factors, such as VEGF, TNF α , and CCL2, derived from the primary tumor up-regulated expression of chemokines S100A8 (see Chap. 15 for overall information) and SAA3, both of which were capable of corecruiting BMDC and tumor cells such as LLC, B16, or E0771 into the lungs [32, 33]. Anti-VEGF antibody alone or in combination with anti-TNF α antibody, anti-S100A8 antibody, CCL2-KO, all abrogated recruitment of BMDC and tumor cells to the lungs. Conversely, pretreatment with VEGF, TNF α , or SAA3 peptides, all enhanced metastasis [32–34]. Thus, we showed evidence (1), (2), and (3) and additionally, the activated paracrine cascade in the lungs toward which tumor cells migrate via circulation.

As judged by Evans Blue leakage assays, conditioned media derived from *in vitro* cultures of tumor cells such as E0771 and LLC (TCM: tumor conditioned medium) showed high potentials to induce vascular permeability per organ weight specifically in the lungs but not kidney, brain, and liver. Subsequent intravenous injection of the PKH26-labeled tumor cells revealed that they were recruited preferentially in the hyperpermeability areas. The TCM contained VEGF and PlGF, and anti-VEGF antibody neutralized the tumor cell recruitment.

What is happening in the hyperpermeability areas?

We looked for receptors that were differentially up-regulated in the higher permeability areas compared with lower in LLC-bearing mice and found CCR2. Conversely, significantly higher expressions of both CCR2 and its ligand CCL2 (MCP-1) were found in the higher permeability areas than in the lower. S100A8⁺ or SAA3⁺ cells were more abundant on the high permeability areas and dramatically decreased in CCL2-KO mice. Conversely, both VEGF and CCL2 individually could induce expression of S100A8 and SAA3, and CCL2 could auto-amplify in lung organ cultures. The lung permeability in tumor-bearing mice was abrogated in either CCR2-KO or CCL2-KO. SAA3 also could activate FAK. In the lungs of tumor-bearing patients, expression of S100A8, S100A9, CCR2, and fibrinogen was higher than those from tumor-non-bearing patients who died by accident [32]. VEGF activated FAK, which in turn induces E-selectin expression. Tumor cell recruitment into the lungs after TCM stimulation was attenuated by either anti-E-selectin antibody or E-selectin-KO background. Conditional overexpression of FRNK, a dominant negative form of FAK, in endothelial cells under the control of tetracycline-responsive element gave the same inhibitory effect on the tumor cell recruitment after TCM. Lung metastasizing tumor cells around the pulmonary vascular bed appear to activate the VEGF/VEGFR2-Src-FAK pathway [34]. Alternatively, S100A8 and SAA3 can activate TLR4, which also can induce FAK activation as just described.

Similar to TAM in primary tumor tissues, Jeffrey W. Pollard proposed an idea of metastasis-associated macrophages (MAM) that are distinct from resident macrophages. CCR2⁺Ly6C⁺VEGFR1⁻ monocytes are precursors of MAM strongly positive in VEGFR1⁺ that secrete CCL3 (Table 2.2 in Chap. 2). Their mobilization from bone marrow to and retention in lungs are dependent on CCR2 and CCR1, respectively [35]. VEGFR1⁺ is unlikely to contribute to their recruitment, but it induces CSF1-mediated autocrine signaling in MAM [36]. Inhibition of MAM recruitment by anti-VEGFR1 antibody, CCR1-KO, or genetic loss of functional CSF-1R resulted in poor efficiency of lung metastasis.

Collectively, it can safely be said that:

1. Primary tumor enhances BMDC recruitment to at least premetastatic lungs. This is partially mediated by tumor-derived VEGF but not VEGFR1 expressed on BMDC. Of note, even in the absence of tumor, BMDC mobilization to the lungs takes place in a homeostatic manner (see Chap. 14).
2. VEGFR1 may modulate BMDC recruitment in postmetastatic lungs without affecting the size and number of the metastatic foci in a tumor species-dependent manner.
3. Tumor-derived factors as represented by VEGF and CCL2, and S100A8 and SAA3 that are induced by the tumor-derived factors in the pulmonary endothelial cells, in other words, endocrine inducers and induced factors, both can activate FAK to exert vascular permeability and E-selectin up-regulation to control entry of BMDC.

13.5.3 Narrow and Broad Sense: The Second Definition of Premetastasis

We need to consider a critical issue, i.e., premetastasis, in a narrow and broad sense. If you suppose a circumstance where lungs do not have any single tumor cell from the primary site, it can safely be called <premetastatic lung in a narrow sense>. However, it is virtually impossible to detect a small number of disseminated tumor cells in any organs even in experimental conditions, let alone in clinical settings. We usually cannot detect tumor cells in any organs by accurate CT scanning until they grow to form nodules at least within the survival period of the patients, although we often can detect circulating tumor cells.

13.5.3.1 Which Come First: Niche Cells or Metastatic Tumor Cells?

Although myeloid cells appear to arrive first in the tissues for future metastasis, we can hardly determine which come first: a small number of tumor cells or myeloid cells for reasons mentioned. Furthermore, it is not only myeloid cells from bone marrow that participate in the development of premetastatic microenvironment. The Rakesh K. Jain group proposed an interesting idea that fibroblasts in the primary tumor also metastasized to distant organs. They showed cotraveling heterotypic tumor-stroma cell clusters in circulation in GFP-mice whose renal capsule was implanted with DsRed-expressing LLC tumor cells. When mechanically dissociated doubly fluorescent tumor fragments from the primary tumor were injected into circulation on purpose, they could observe metastasis in the lungs. Importantly, the metastatic nodules contained stromal cells derived from the primary tumor. Colocalization of GFP with SMA, fibroblasts-specific protein 1, and F4/80 was detected in 80 %, 75 %, and 28 %, respectively. Clinical samples of brain metastasis also contained SMA⁺ cells, whereas normal brain lacks fibroblasts [37]. Those results imply the possibility that not only myeloid cells but also fibroblasts may travel alone or with tumor cells from the primary tumor to the distant metastatic tissues.

The Russel S. Taichman group showed that murine prostate cancer RMI cells secrete CXCL16 (see Chap. 2), which stimulates MSC via CXCR6 to induce conversion from MSC to CAF that interacts with the tumor cells through CXCL12 produced in CAF and induced CXCR4 on the tumor cells [38]. This promoted their bone metastasis as assessed by RFP-labeled RMI cells that were subjected to intracardiac injection in the presence or absence of plerixafor (also called AMD3100), a CXCR4 inhibitor, or of CXCR6 knockout condition in the injected mice.

13.5.3.2 Metastasis Dormancy

If you assume conditions where a small number of tumor cells are disseminated in an organ that can be hardly detected, it should be called <premetastatic organ in a broad sense>. The idea that a certain population of disseminated tumor cells stays quiescent before regrowth brings attention to this issue. In this case, premetastatic microenvironment is a condition that fails to allow growth of the disseminated tumor cells.

Clinically, it is called metastasis dormancy. It is needless to say that the microenvironment is called postmetastatic after regrowth, which is similar to primary tumor microenvironment. I will show two premetastatic experiments in a broad sense.

TSP-1 Keeps Quiescence in Vascular Niche

Orthotopic injection of spontaneously metastatic MDA-MB-231 breast cancer cells expressing GFP-luciferase or HMT-T4-2 breast cancer cells expressing mCherry into inguinal mammary glands of NOD-SCID mice resulted in tumors, which were resected at 3 weeks after injection with no sign of relapse until 6 weeks. Bioluminescence analysis revealed that GFP-positive tumor clusters were closely localized on CD31-positive endothelial cells in lungs and bone marrow and appeared to be quiescent as judged by negative Ki-67, whereas resected tumors showed highly positive Ki-67 [39].

Serum- and cytokine-free coculture of YFP-expressing T4-2 cells with pulmonary fibroblasts in the presence or absence of HUVEC-E4ORF1 (see Chap. 11 for HSC niche) expressing mCherry revealed that 77.4 % of T4-2 cells remained negative in Ki-67 for 17 days with HUVEC-E4ORF1. Given that MSC from bone marrow gave 88.1 % of Ki-67-negative tumor cells in the same experiment, microvascular niches may be established in the lungs to keep the tumor cells in a dormant or quiescent state in a similar fashion to HSC quiescence in bone marrow. Comparative proteomics identified TSP-1 that was derived from the vascular niche. Readers should remember the antagonist between VEGF and TSP-1 (see Chap. 11). TSP-1 alone could recapitulate the tumor growth-suppressive niche function for HUVEC-E4ORF1. This inhibitory activity is released by vascular sprouting with neovascular tip cells proficient in TGF β , periostin, tenascin, versican, and fibronectin and deficient in biglycan and LTBP1 (latent TGF β -binding protein 1). Another researcher identified perlecan, heparin sulfate proteoglycan produced by endothelial cells, as a potent inhibitor for growth and invasion of MDA-MB-231 breast and A549 lung cancer cells [40].

VCAM-1 on Tumor Cells Releases Quiescence

An *in vivo* selection strategy to establish and subsequent microarray analysis between highly and poorly bone metastatic sublines from MDA-MB-231 breast cancer cells identified VCAM-1 as a prometastatic gene product (see Fig. 11.4 in Chap. 11). VCAM-1 expressed in cytokine-activated endothelial cells is a ligand for integrin $\alpha 4 \beta 1$ (very late antigen-4 or VLA-4) on leukocytes, the binding of which plays an important role in their adhesion and transendothelial migration. CD11b⁺ cells from bone marrow contained $\alpha 4 \beta 1$ cells that differentiated into osteoclasts under CSF-1 and RANKL treatment. Anti-VCAM-1 blocking antibody inhibits and enforced VCAM-1 overexpression in MCF7 breast cancer cells enhances bone metastasis in this model. VCAM-1 is dispensable for survival and initial growth of dormant micrometastatic tumor cells. Authors suggest VCAM-1 expression in quiescent tumor cells is incidental but dependent on sustained NF κ B signaling of undetermined origin. NF κ B also can induce expression of GM-CSF, CXCL1, and CSF-1, all of which can facilitate bone metastasis [41].

Antitumor Activity at Premetastatic Sites

In primary tumors, both growth- or survival-promoting and -inhibiting activities are present. While VEGF from M2-polarized TAM and N2-shifted TAN favor tumor growth by tumor angiogenesis, CD8⁺ T cells manage to attack the tumor cells and N1-switched TAN potentially kill tumor cells by TNF α and ROS. A similar coexistence of antagonizing activities is found in premetastatic lungs.

The Robert Benezra group in 2011 showed it in the orthotopic 4T1 tumor model with spontaneous metastasis preferentially to lungs as compared with liver or brain [42]. They implanted puromycin-resistant 4T1 cells, and lungs with potential metastasis were dissociated by collagenase and plated back into liquid culture containing purimycin for 14 days to quantitate the tumor cell colony. The colony numbers of roughly 10 and 70 were observed at 7 and 14 days post-engraftment, and they assumed premetastatic lungs at day 7 even if they could take tumor cells from the lungs.

CCL2 derived from 4T1 tumor cells <entrained> neutrophils to produce ROS through neutrophil Nox2, which is supposed to kill directly the tumor cells, as in the case of N1-favored TAN [42]. This tumor-killing activity is modified in an organ-specific manner. In the primary 4T1 tumor, TGF β is abundantly produced to counterbalance the activity similar to N2. However, TGF β is deficient enough in the lungs before metastasis to allow CCL2-stimulated neutrophils to make an entry into the lungs to counterattack the arriving tumor cells. This also could happen in athymic mice, indicating that the phenomenon was independent of T cells. Neutrophils purified from 4T1-bearing mice delayed formation of metastatic foci in the lungs. In fact, they showed that Ly6G-mediated neutrophil depletion increased metastatic burden of the tumor cells until 14 days post-engraftment without changing (1) the primary tumor growth, (2) the CTC numbers, and (3) the metastatic activity to liver and brain.

I underline an important point here. They observed no gross tissue damage in the premetastatic lungs even in the presence of numerous neutrophils. One critical difference from our system is that they could hardly observe vascular permeability in the premetastatic lungs.

Dormancy-Inducing Activity as an Anti-tumor Effect

The Filippo G. Giancotti group has discovered Coco in 2013, which is a secreted antagonist of BMP and reactivates the growth of quiescent metastatic cells by blocking the dormancy-inducing activity of BMP derived from host lungs [43]. Coco appears to shield the tumor cells by binding surrounding proteoglycans to counteract against BMP. Coco expression in 4T07 cells that were subjected to orthotopic injection into the mammary fat pad promoted spontaneous metastasis in the lungs but not bone or brain.

13.5.4 Dissection of Cell Mobilization and Entry via Endothelial Barrier

Tumor-derived factors as represented by VEGF, PlGF, and G-CSF causes mobilization of myeloid cells from bone marrow, which make an entry into the premetastatic tissues before the actual arrival of tumor cells. The Amato J. Giaccia group focused on lysyl oxidase (LOX) [44].

LOX is an amine oxidase engaged in the oxidative deamination of lysine residues in collagen and elastin yielding H_2O_2 , ammonia and aldehyde residues on the substrates [45]. The spontaneous reaction of the aldehyde residues results in fiber cross-linking to maintain their structure and tensile strength in a homeostatic manner. LOX-KO mice die perinatally due to aortic aneurysm, cardiovascular dysfunction, and maldevelopment in respiratory system with fragmentation of elastic fibers [46].

Synchronously with the covalent cross-linking LOX is inactivated syncatalytically by the products H_2O_2 and ammonia to avoid over-crosslinking. Tumor-derived LOX as a result of hypoxic response is secreted, proteolytically processed by an as yet unknown mechanism to an active form capable of binding to fibronectin with a high affinity and degrading collagen type IV but not fibronectin. In premetastatic experiments with LLC or orthotopic implantation of human MDA-MB-231 breast cancer cells in nude mice, fibronectin is up-regulated possibly by fibroblast-like stromal cells within 7 days after tumor challenge with which LOX is colocalized [44]. CD11b⁺ myeloid cells when attached to fibronectin up-regulate expression of MMP2 while MMP9 activity remains constant. Collagen IV in the endothelial basement membrane is degraded as judged by in vitro experiments with Matrigel incubated with CD11b⁺ cells releasing collagen type

IV peptides in a MMP2-MMP9 inhibitor (tissue-permeable cyclic peptide CTTHWGFTLC)-sensitive manner. Peptides released from collagen degradation appear to have dual functions. Some of them are inhibitory in angiogenesis and others promoting. One of their interesting results was that the induction of active MMP 2 was dependent on contact of CD11b⁺ cells with collagen IV preincubated with purified LOX in an anti-LOX neutralizing antibody-sensitive manner. LOX is induced in hypoxia. The physiological role may be to activate transendothelial cell migration of leukocytes by intensifying the tensile strength of basement membrane, which concomitantly induces their MMP2 expression to further facilitate the event.

The byproduct H₂O₂ also might activate Src/FAK to cause vascular permeability and an antiapoptotic effect of NH₃ against TNF α is stated in Chap. 10.

Hypoxia in primary tumors pathologically disturbs this homeostatic phenomenon before the arrival of tumor cells in distant organs.

In postmetastatic human samples, including liver, brain, and lymph nodes, the authors found CD11b⁺ cells in 51 of 95 lesions typically associated with LOX⁺ staining [47]. An LOX inhibitor β -aminopropionitrile (BAPN) had no effect on the postmetastatic tumor growth but significantly reduced metastasis when started to administer one day before intracardiac injection of breast cancer cells [48].

13.5.5 Premetastasis Without Primary Tumor

I stated that forced intravenous injection of tumor cells causes lung metastasis without establishing any primary tumors and genetic analysis of clinical samples revealed that metastatic clones are present in the primary tumor. However, given that oncogenes are required only after addiction to them for growth or survival, it is likely that metastasis can occur at any time point during tumor progression, including untransformed, seemingly normal, and mutation-acquiring initiation stages. The Harold Varmus group dissected the seeding process from malignant transformation by intravenously injecting dissociated cells from the mammary gland with tetracycline (Tet)-inducible expression of Myc and K-RasD12 in a mammary-specific manner that guarantees the transformation process followed by Tet to induce lung metastatic tumors [49]. Orthotopic xenografts in Rag1-KO mice caused lung metastasis whose pathological findings were similar to those induced Tet. They found similar phenotypes in another group of mice in which mammary gland-specific expression of PyMT with firefly luciferase to track down the cells. The mammary cells before Tet induction remained inconspicuous and untransformed for 120 days. Suppose that life spans of mouse and humans are 800 days and 80 years, respectively, the 120 days correspond to 12 years in human life.

Intravenously injected tumor cells can settle down in the lungs in tumor-non-bearing mice but the efficiency increases in tumor-bearing mice. This raises questions of whether lung metastatic niches are preformed (premetastatic niches in tumor-bearing conditions) before or postformed after tumor cell arrival, and niches

occupied by lung stem cells in physiological circumstances also are utilized by metastasizing tumor cells. Preformed and postformed niches are not mutually exclusive. In both cases, primary tumor cells affect their establishment in an endocrine or paracrine manner, respectively. If primary tumors exist, some of them, but not others, can drive the formation of lung premetastatic niches [33]. However, we cannot completely rule out the possibility of tumor cell-driven premodification of the lungs even after intravenous injection of tumor cells in tumor-non-bearing mice (see Fig. 16.3 in Chap. 16 for the modes of metastasis experiments). The injected tumor cells may produce certain molecules during their circulation in the blood stream that can enhance tumor cell entry and settlement in the lungs before their arrival.

Here I remind readers of an analogy between LPS-stimulated normal HSC (Chap. 11) or CML-HSC (Chap. 9) metastasizing from bone marrow to spleen and cancer cells metastasizing from kidney for example to lungs (see Fig. 11.4 in Chap. 11).

We still lack evidence that lung stem cell (LSC) niches can serve as preformed niches for metastasizing tumor cells. However, a competitive replacement has been reported in prostate cancer cells that directly compete with HSC to obtain occupancy in HSC niches resulting in bone metastasis [50] (Fig. 13.4). In this case, native HSC niches that may or may not be influenced by the primary tumor serves as premetastatic niches and are squatted. Upon arrival of tumor cells they may stimulate and change the adjacent areas to maintain their settlement in the fully established metastatic niches. Distinct preformed lung niches have been assumed whose components include recruited cells from bone marrow and resident cells, such as club cells and alveolar macrophages (Fig. 13.4).

Interstitial space is the major site of lung metastasis, particularly areas adjacent to the terminal bronchioles in which club cells are located [51, 52]. In a lung stem cell model, human lung Lgr6⁺ stem cells (LSC) can differentiate to mature bronchioalveolar epithelial cells when injected under the kidney capsule of nude mice by recruiting and interacting with fibroblasts. The reciprocal paracrine signaling between LSC and fibroblasts is established in the interstitial space. LSC-produced CXCL12 stimulates CXCR4 on the fibroblasts, which in turn secrete TNF α . TNF α induces an autocrine loop of TGF β 1 in LSC leading to CXCL12 expression [53]. This suggests that LSC niches are formed after LSC injection and their formation is initiated by recruiting fibroblasts in a CXCL12-CXCR4-dependent manner.

An important unsolved question is how metastasizing tumor cells are selected against harsh conditions in other organs distinct from their primary sites. Negative selection pressures on growing tumor cells might give them stem cell-like properties under duress. Stress granules that contain translationally stalled mRNA preinitiation complexes are observed in those conditions, reduction of which by knockdown of RNA-binding proteins like G3BP-1 curbed lung metastasis by human sarcoma cell xenografts [54]. This suggests that defective strategy for triage in noxious microenvironments can inhibit tumor progression.

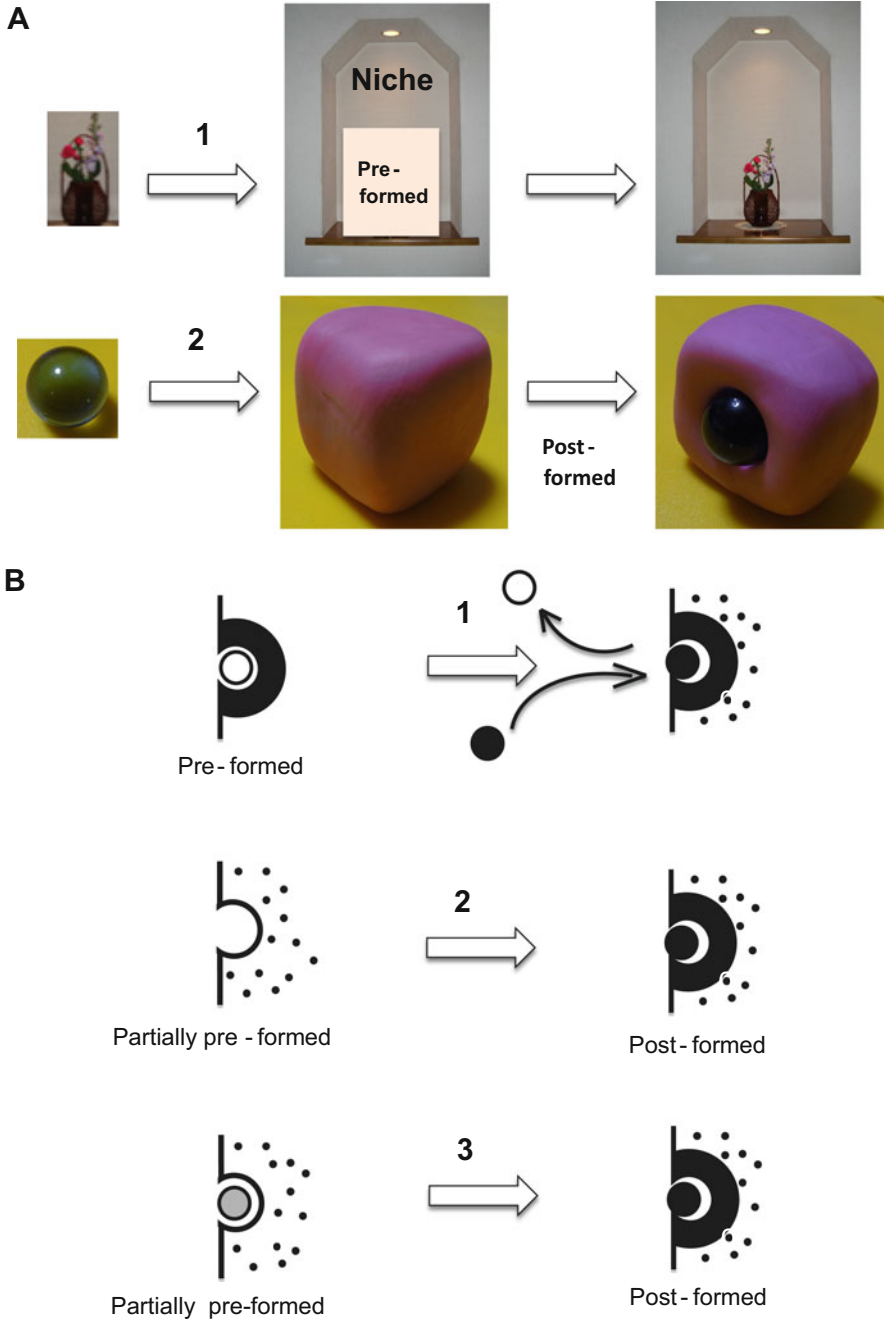


Fig. 13.4 Images of plasticity of niches. (A) Niches originally mean a recess within a wall to put a flower vase. In this case, the niche is preformed [1]. A ball hitting and making a self-occupying dent in clay is an image of a postformed niche [2]. (B) HSC (white circle) appears to occupy preformed native HSC niches in bone marrow in which tumor cells (black circle) can competitively squat [1]. Premetastatic lung niches may be partially formed before the arrival of tumor cells

Cancer stem cells (CSC) (see Chap. 11) efficiently cause lung metastasis when injected intravenously as exemplified in the case of CD90⁺CD24⁺ CSC in the MMTV-PyMT mouse breast cancer model [55], suggesting that they can tolerate with the tissue microenvironment different from the original organ. When a strategy was taken to impair autophagy in tumor cells (see Chap. 10), release of dormancy, i.e., regrowth was observed in an example of ARHI (aplasia Ras homolog member I), alternatively called DIRAS3. Metastatic progression of ovarian cancer to peritoneal cavity by activated migration correlated with reduced ARHI expression levels, reexpression of which not only inhibited FAK-Src-mediated RhoA activation for migration but also promoted autophagosome maturation in a manner involving mTOR down-regulation and FOXO3a nuclear retention [56, 57]. The ARHI was shown to induce autophagy as well as tumor dormancy in a xenograft model of SKOV3 ovarian cancer cells in which ARHI expression was regulated by a Tet-on inducible system. This was abrogated by chloroquine. Given that chloroquine reduced regrowth of xenograft tumors that took place with forced reduction of ARHI expression levels, autophagy is likely to support survival of dormant tumor cells such as CSC [58].

13.5.6 Premetastatic LN

IL-6 produced by triple-negative breast cancer stimulated lymphatic endothelial cells (LEC) not only in the premetastatic lungs but also in the premetastatic lymph nodes through Stat3 and HIF-1 α , which in turn secreted CCL5 to recruit the tumor cells to those tissues [59]. Sentinel lymph nodes also were reported to be cultivated as soil for metastatic tumor cells as seed by tumor-derived exosomes. Preinjection of DiR-labeled exosomes derived from B16-F10 melanoma cells enhanced recruitment of subsequently injected DiO-B16 melanoma cells around exosome-rich areas in sentinel lymph nodes [60]. Highly expressed genes in the exosome- relative to control liposome-injected preparation of sentinel lymph nodes included stabilin1, α v β 3 integrin, TNF α , HIF-1 α , and so forth. Conductive soil for seed is established.

Expression of VLA-4 is necessary and sufficient for lymphatic metastatic progression of LLC. Intradermal injection of VEGF-C failed to induce lymphangiogenesis in mice with Y991A inactivating mutation in α 4, conditional α 4 knockout in lymphatic endothelial cells, or a small molecule α 4 β 1 inhibitor called ELN476063 and lymph node metastasis of LLC that were injected into lymphatic vessel-rich footpads. They showed that VEGF-C stimulated PI3K-



Fig. 13.4 (continued) by nontumor cells (indicated by dots), i.e., recruited cells, such as bone marrow-derived myeloid cells and/or resident cells, including alveolar macrophages and club cells, and the niches are completed with tumor cells (postformed) [2]. Pulmonary recruitment of untransformed ectopic cells (gray circle) partially forms niches for tumor cells to occupy after their arrival [3]

dependent expression of VLA-4 that prepares a soil for VCAM-1-expressing tumor cells to come and settle [61].

NKT cells recognize glycolipids presented by CD1d. While CD1d is absent in many tumors, it is expressed in TAM as exemplified in those in human neuroblastomas. In the xenografts in NOD/SCID mice, TAM-secreted IL-6 activated the tumor growth. In this experimental condition, NKT targeted CD1d-positive TAM but not CD1d-negative tumor cells [62]. It remains to be elucidated that myeloid lineage cells that participate in the formation of premetastatic niche are NKT targets.

13.5.7 Triangle of Premetastasis (Fig. 13.5)

In an excellent review by the Andreas Moller group on premetastatic niche, they referred to four points in niche formation: (1) ECM remodeling, (2) immunosuppression, (3) inflammation, and (4) vascular hyperpermeability [63]. In my thinking, both vascular permeability and immunosuppression by recruited leukocytes are features of inflammation, and the expression of fibronectin and degradation of type IV collagen are accompanied by leukocyte mobilization.

I remind readers of the idea of triangle consisting of inflammation, angiogenesis, and coagulation as discussed in both Chaps. 3 and 12. As I defined frequently in this book, inflammation basically fulfills cell mobilization through the blood stream. It is no wonder that inflammation can “metastasize”, by which premetastatic microenvironment is cultivated. The inflammatory signals, such as secretion of VEGF and TNF α , in melanoma B16-bearing mice induce expression of MMP3, MMP10, and Ang2 (see Chap. 3) in the premetastatic lungs to result in vascular instability leading to vascular permeability-stimulated activation of coagulation cascade with final fibrin deposits as confirmed by electron microscopy [64]. This is dependent on the vascular permeability factors VEGF and TNF α . Fibrin clot and tumor cells interaction mediated by plasma fibronectin bound to fibrin and $\alpha v \beta 3$ on tumor cells facilitated extravasation in the lungs.

Suppose that one of the early events in premetastatic lungs is vascular permeability by remote controls of the primary tumor. An element(s) produced from the primary tumor, such as VEGF, TNF α , and CCL2, should render the lungs hyperpermeable. We have shown that well-known permeability factors, VEGF, TNF α , and CCL2, when individually injected into the peritoneal cavity immediately followed by intravenous injection of Evans Blue induced an increased vascular permeability in the lungs, which was peaked at 3–5 h after the permeability factor administration [32]. As stated earlier, increased fibrin-fibrinogen deposits were observed in the lungs of tumor-bearing patients [32]. The two up-regulated factors in the lungs, S100A8 and SAA3, also gave similar levels of vascular permeability in the lungs. In addition, anti-VEGF or anti-TNF α neutralizing antibody could block up-regulation of S100A8 in the lungs. S100A8 can promote

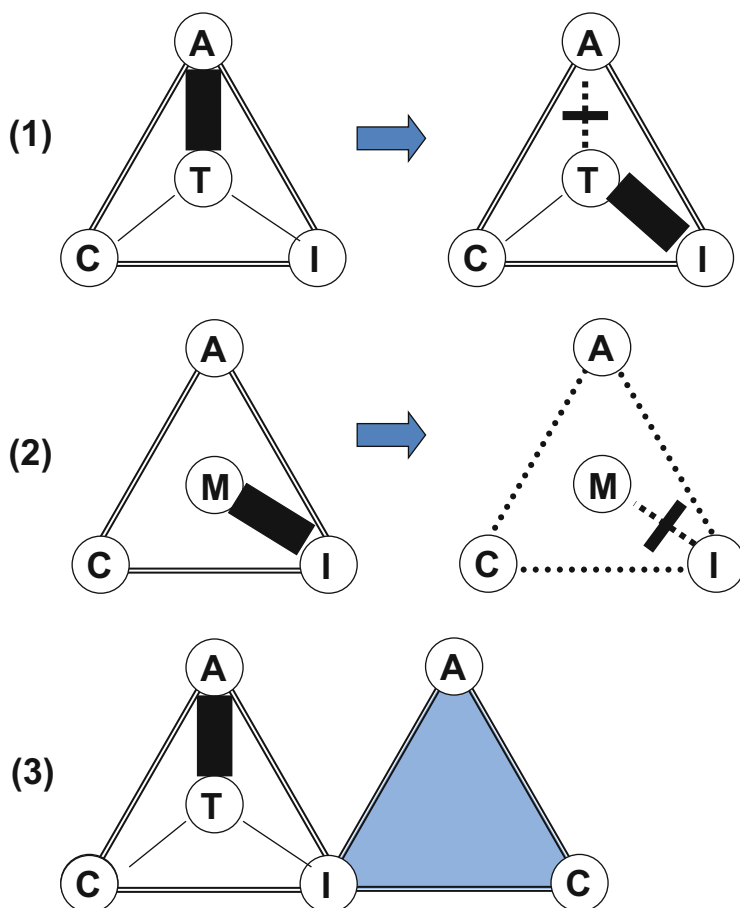


Fig. 13.5 Triangle of premetastasis. (1) Primary tumor (T)-produced VEGF drives tumor angiogenesis (*thick bar*). When tumor angiogenesis (A) is blocked by VEGFR2 inhibitors, inflammation (I) is activated by the resulting necrotic tumor tissues, which persistently supports the triangle stability giving resistance to anti-VEGF therapy. Coagulation (C). Also see Fig. 3.6 in Chap. 3. (2) Microbes (M) induce inflammation (I) through pattern recognition receptors. However, total killing of the microbes can eliminate M-to-I signaling, which destroys the stability of the triangle. (3) Primary tumor-associated inflammation can spread to other organs that still lack tumor cells, where a new triangle may be established providing premetastatic soil before the arrival of metastasizing tumor cells

expression of SAA3 in both macrophages and endothelial cells. The blood levels of S100A8 and SAA3 also significantly increased roughly 8–11 days after the primary tumor implantation. Moreover, CCL2 can activate the S100A8-SAA3 cascade in the lungs. We have shown that both S100A8 and SAA3 are endogenous ligand of TLR4, thus stimulating mobilization and pulmonary entry of TLR4-expressing bone marrow-derived CD11b⁺ cells [65].

The David Lyden group showed that PKH67-labeled exosomes of 100 nm in size (see Chap. 11 for microvesicles) that were derived from B16-F10 melanoma reached the lungs, bone marrow, liver, and spleen 24 h after intravenous injection. At this time point, Texas Red-labeled dextran was injected to confirm the increased endothelial permeability, presumably caused by S100A8, S100A9, and TNF α whose expressions were up-regulated in the lungs after the exosome delivery [66].

Given the presence of bioactive exosomes or larger microvesicles derived from tumor cells not only in tumor conditioned medium (CM) *in vitro* but also in the serum of tumor-bearing mice, we should be careful to interpret the experimental results obtained with those materials, i.e., CM and serum. For example, we have stimulated lungs cultured *in vitro* with serum from LLC tumor-bearing mice and observed up-regulation of S100A8 in the lungs in a manner at least partly dependent on VEGF and TNF α in the serum [33]. However, existence of full-length EMMPRIN (extracellular MMP inducer, also called basigin or CD147) in the microvesicles of 300–400 nm in size that were shed from lung cancer NCI-H460 cells was shown and the release was augmented by PMA, an activator of protein kinase C in a dose- and time-dependent fashion [67]. The microvesicles *in vitro* underwent breakdown in 16 h, but the EMMPRIN proteins were still in the supernatant. Of note, EMMPRIN has been shown to be a receptor for S100A9.

Proteomics analysis of tumor exosomes revealed that expression pattern of integrins could predict metastatic organs in which corresponding resident cells participated in the fusion with the integrins [68]. Redirection of exosomes was possible with the matching information. Integrin-mediated precise signaling in terms of the true determinants for organotropism awaits further investigation.

13.6 BMDC are affected by Tumor-Derived Factors

The vascular leakiness coincides with entry of BMDC into the pulmonary interstitium. Generally, BMDC that consists of various types of cells never guarantees premetastatic lungs and some of them may be a symptom but not a cause of premetastasis. Given that the concept of premetastasis has not been established completely, as I have expanded the premetastasis idea to the organs with dormant tumor cells in this section, metastasis experiments often are described as a mixture of pre- and postmetastasis without notice. For example, physical presence of BMDC persists in pre- through postmetastasis, but its function may change over time.

When bone marrow cells of FVB transgenic mice with GFP were used to reconstitute the marrow of FVB/n mice, 12.4 % of total lung cells were GFP⁺, 6.4 % of which were CD11b⁺Gr1⁺ cells. MMTV-PyVT mice under the same procedure of BMT gave 36.3 % (threefold) of GFP⁺ cells in the lungs. CD11b⁺Ly6Chigh monocytic cells were found to produce versican [69]. MMTV-PyMT-recipient mice that underwent BMT with Lin-bone marrow progenitor cells infected by lentiviruses expressing versican-specific shRNA gave lungs recruited by an unaltered number of BMDC but with decreased versican levels and suppression

of metastasis. The authors claimed that versican promoted mesenchymal to epithelial transition (MET) since purified versican at 2.5 $\mu\text{g/ml}$ increased expression of both E-cadherin and occludin and decreased vimentin in MDA-MB-231 cells, and thereby facilitated growth of metastatic tumor cell growth. They also provided clinical information that versican was detected around the CD11b⁺ myeloid cell clusters in the postmetastatic lungs, and its levels in the lungs and liver were roughly sixfold each over those in normal organs [69].

The Michael Karin group, on the other hand, purified versican from LLC cell-conditioned medium and showed that versican activated the TLR2-TLR6 complex with subsequent induction of TNF α , IL-1 β , IL-6, CCL3, CCL4, CXCL2, and CXCL1. Both TLR2 and TNF α were required for chemokine induction in the lungs and for LLC to accomplish metastasis [70]. It can be assumed that primary tumor-derived versican may serve as a triggering mechanism to induce pulmonary recruitment of versican-secreting BMDC as a second step, which thirdly amplifies chemokines resulting in expansion of BMDC in the lungs and recruitment of TLR2-expressing tumor cells. Premetastasis includes up to the second step and upon arrival of tumor cells that also produce versican metastasis is accomplished.

Another group stimulated CD11b⁺Gr1⁺ cells isolated from normal C57BL/6J mice with exosomes derived from in vitro culture of B16 melanoma cells, in which they found no versican, resulted in production of IL-6, TNF α , and CCL2 in a manner dependent on MyD88 [71]. It also was true in vivo. Intravenous administration of the exosomes twice a week for 3 weeks enhanced accumulation of CD11b⁺Gr1⁺ cells in the lungs and liver in the wild-type C57BL/6J but not MyD88-KO mice. Those CD11b⁺Gr1⁺ cells isolated from the exosome-treated, wild-type mice suppressed anti-CD3 mAb-stimulated T-cell proliferation [71].

The David Lyden group analyzed the proteomic profile of the B16-F10 exosome, found Met (HGF receptor), CD44, and Hsp70, etc. and claimed that the exosome-mediated horizontal transfer of Met to BMDC, which is then sensitized to HGF-stimulated signaling resulting in their mobilization from bone marrow [66]. BMT of C57BL/6 mice with bone marrow from GFP mice that had been treated with B16-F10 exosomes for 4 weeks increased BMDC recruitment and vascular density by roughly 4.5- and 3.0-fold, respectively, in the primary tumor. Furthermore, the exosome release was shown to be dependent on Rab27, knock-down of which in B16-F10 cells diminished the primary tumor growth as well as lung metastasis [66]. Thus, the primary tumor-derived exosomes can sensitize BMDC that facilitates tumor progression in both primary sites and metastatic, presumably also premetastatic, sites. The same group showed that uptake of exosomes derived from pancreatic cancer cells by liver Kupffer cells induced TGF β secretion and subsequent production of fibronectin, to which BMDC are recruited to form hepatic premetastatic niches [72]. It is not surprising that inhibition of BMDC affects both primary and metastatic sites.

What is the most fundamental affair in the primary tumor that can remotely control the bone marrow cells? Hypoxia is one of the unique features of primary tumor [73]. Conditioned culture medium from E0771 cells under hypoxic condition of 2 % contained increased levels of VEGF, TNF α , G-CSF, and CCL2 compared

with that from normoxic condition of 20 %. Daily injection of the medium for 7 days, which mimics stimulation by the primary tumor without its actual presence, therefore serves as premetastatic conditioning, resulted in pulmonary recruitment of CD11b⁺Ly6C^{med}Ly6G⁺ cells, which could be abrogated by anti-CCL2 antibody, suggesting that one of the most potent factors is CCL2 [74]. Then, why do CCL2-stimulated bone marrow cells reach the lungs? Here you can find a fundamental issue. It is clear that the primary tumor is an inflammatory tissue in which vascular permeability is increased and various types of leukocytes come in. Factors derived from the primary tumor cells, such as CCL2, stimulate not only bone marrow cells but also lung endothelial cells to initiate the S100A8-SAA3 paracrine cascade as one pathway. Both VEGF and TNF α also induce pulmonary S100A8 and SAA3, causing increased vascular permeability. Fibronectin is shown to be up-regulated. Bone marrow-derived cells in the lungs express the receptors for those ligands; TLR4 for S100A8 and SAA3, VLA-4 for fibronectin. Therefore, it appears that the inflammation in the primary tumor “metastasizes” to the lungs before the actual arrival of metastatic tumor cells.

Premetastatic lungs have not only CD11b⁺Gr1⁺ cells but also CD3⁺NK1.1⁺ CD11b⁺CD27^{low} immature NK cells. NK cell maturation to CD11b^{high}CD27^{low} cells is likely to be blocked, thereby facilitating metastatic growth by suppression of the antitumor activity of NK cells [74]. It is interesting that NK cells are activated in the lungs by the expanded CD11b⁺ Gr1^{med}Ly6C⁺Ly6G[−] cells producing CCL5 and CXCL9 3 days after intravenous injection of B16 melanoma cells into NLRP3-KO mice, resulting in reduced metastasis. Although this tumor challenge cannot be a true premetastatic condition, because the injected B16 cells could reach the lungs during the period of 3 days, NLRP3 activation might inhibit the expansion of the CD11b⁺ Gr1^{med}Ly6C⁺Ly6G[−] cells and the endogenous TLR4 ligands might activate NLRP3 as in the case of LPS [75].

As I stated in the clinical information section in this chapter, presence of tumor, whether dormant or growing, causes inflammation. Early elimination of primary renal cell cancer is quite reasonable. Manifestation of pulmonary metastasis after nephrectomy indicates the persistent presence of dormant tumor cells in the lungs or somewhere else in the body, which manifest themselves by starting to regrow. During the in-between period, the lungs are cultivated as premetastatic soil before regrowth. The metastatic lung tumors, for example, in right lung further prepare the soil in the left lung. Metastasectomy of the right metastatic lung tumors may decrease the cultivation. It may appear endless like the chicken and egg; which comes first, premetastatic milieu or tumor cells?

13.7 Pseudo-Hypoxia and Pseudo-Starvation in Inflammatory Cells

As stated earlier, hypoxia in primary tumors induces elements, including CCL2 and LOX, that can reach lungs via circulation, which contributes to the establishment of premetastatic milieu that allows entry of metastasizing tumor cells. Does hypoxia exist in premetastatic lungs? Pimonidazole incorporation in premetastatic lungs is almost negative in our hands (unpublished results). Dissociation among hypoxia, perfusion, and HIF-1 α up-regulation as judged by pimonidazole, Hoechst33342, and anti- HIF-1 α immunostaining, respectively, was reported in the orthotopic 4T1ODD-luc tumor model treated by doxorubicin [76]. This suggests hypoxia-independent activation of HIF-1 α and subsequent induction of HIF-1 α target genes. Nonhypoxic conditions, such as nitric oxide generation by VEGF-activated eNOS, also can lead to PHD inhibition-mediated activation of HIF-1 α at least transiently [77]. Premetastatic niche cells, especially prometastatic immune cells, such as BMDC and macrophages, may be under pseudo-hypoxia. Given the reciprocal activation between NF κ B and HIF-1, NF κ B activation induced by TLR4 stimulated by endogenous ligands, such as S100A8 and SAA3, could cause pseudo-hypoxia (see Chap. 11). In response to increased AMP/ATP ratio during up-regulated glycolytic pathway under hypoxia, AMPK is activated. The opposing effect of AMPK is proposed to have anti-inflammatory consequences in those metabolically transformed immune cells [78].

13.8 Spatial Issue

In our premetastatic model, the assumed primary tumor is developed by implantation either orthotopically (for example, breast cancer cells in mammary fat pads) or ectopically (lung tumor cells in subcutaneous tissues), and a target organ is analyzed, because those two distantly located tissues are interconnected by circulation. The same situation can be found between the right-sided lung or brain hemisphere and the left-sided. Even within the right-sided lung with tumors, tumor cell-free portions of the right-sided lung are of the same organ or tissue and of the same side and connected not only by contiguous stroma but also by circulation.

Roughly 50 % of metastatic brain tumors originate from lung tumors [79]. Whereas both organs have continuous nonfenestrated endothelial cells partially covered by pericytes and basement membrane, brain endothelial cells that are closely associated with endfeet of astrocytes have tight junctions and poor pinocytic activity, and express P-glycoprotein, thereby forming the blood-brain barrier (BBB) against chemicals, including pharmaceutical drugs [80].

This vascular integrity is disrupted in brain tumors, such as glioblastoma, as in the case of many other invasive tumors. The vascular state, including FITC-dextran-monitored vascular permeability, and association with astrocytes in an

orthotopic xenograft model of brain tumor could be partially restored back to normal in FAK conditional knockout mice doubly crossed with mice with tamoxifen-inducible Cre activation under the control of the endothelial cell-specific Tie2 promoter and backcrossed into Rag2-KO immunodeficient mice [81]. This indicates that both premetastatic lung (see above for details) and brain tumors have a similar FAK-dependent vascular permeability condition for tumor progression.

Can premetastatic microenvironment be established in the brain? Intracranial injection of highly metastatic Lewis lung carcinoma 3LL cells into the right hemisphere of BALB/c mice exhibited hyperpermeability in the tumor whose level was correlated with the tumor growth and vascular volume as judged by ^3H -mannitol assays and gadolinium-enhanced MRI during the mean survival time of 17 days [82]. Surprisingly, there was no increase in vascular permeability on the left hemisphere, suggesting that even the presence of highly metastatic lung tumor within the same organ may not be able to disrupt the tight BBB integrity through the remote control. The persistent presence of the BBB, if any, may confer resistance to antineoplastic drugs. However, tumor cell injection through the internal carotid artery in nude mice induced the forced brain metastasis. LPS administration in the right lungs enhanced tumor cell recruitment in the lung of the same side (Hiratsuka and Maru, unpublished data). Thus, although I described that increased vascular permeability is required for tumor cell metastasis in the case of lungs, it depends on the metastatic organs.

13.9 Concluding Remarks

Physical evidence is accumulating to show that premetastatic organs actually are established before the arrival of metastasizing tumor cells from the primary site. Suppose premetastatic niches are preformed, spreading of inflammation, which is present within the primary tumor, to the premetastatic organs may contribute to their establishment.

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Chapter 14

What Is Homeostasis?

14.1 Milieu Intérieur

I believe that the idea homeostasis originates from what was described as <milieu intérieur, internal milieu> by Claude Bernard in 1865 [1] (see Table 9.1 in Chap. 9).

“Chez tous les etres vivants le milieu intérieur, qui est un veritable produit de l’organisme, conserve des rapports necessaires d’échanges et d’équilibres avec le milieu cosmique exterieur; mais, a mesure que l’organisme devient plus parfait, le milieue organique se specialize et s’isole en quelque sorte de plus en plus du milieu ambiant.”

[In all lives the internal milieu, which is certainly produced by the organism, conserves the relationship required for exchange and equilibrium with the external world; however, the organism becomes more complete, it is more independent of the surrounding environment. Translated by Y. Maru].

If the organism is a mammalian cell or an organ in a body, they cannot survive alone and require signals for living from growth factors of endogenous origin and nutrients mainly of exogenous supply. Given that most of the food-supplied nutrients are metabolites synthesized by the host, they also can be called endogenous molecules except for special substances, such as vitamin B12, that humans cannot generate (see Chap. 5). Thus, homeostasis in mammalian cells is practically maintained by endogenous molecules.

14.2 Homeostasis at Different Levels

14.2.1 Whole-Body Level

14.2.1.1 Thyroid

The most representative example under homeostatic control may be an oscillation of human thyroid function (Fig. 14.1). In the hypothalamus-pituitary-thyroid axis, thyroid-stimulating hormone (TSH) secreted from pituitary stimulates thyroid to secrete thyroid hormones, such as T3 (tri-iodothyronine). The TSH secretion is suppressed by T3 by the mechanism in which T3 binds the thyroid hormone receptor isoform β_2 , which interacts with the thyroid hormone responsive elements on the TSH promoter [2]. This defines the pituitary-thyroid feedback system to maintain the levels of both hormones within certain ranges. In hyperthyroidism, persistently high levels of T3 and T4 and low levels of TSH are observed. Different from the rather sensitive control of the hormone levels by the feedback system in a healthy condition, it could take up to 6 months for the plasma levels of TSH, whose half-life is just 1 h, to be restored to normal in acute thyroiditis with transiently elevated levels of T3 and T4 whose half-life is 1 and 7 days, respectively. Conversely, transsphenoidal resection of TSH-producing adenoma that constantly stimulates the thyroid to secrete T3 and T4 to induce hyperthyroidism can ameliorate the hyperthyroidism [3]. It is likely that once the long-lived negative feedback normally responsible for the fine-tuning of the homeostatic levels is disrupted, the repair takes time.

14.2.1.2 Neutrophils

Suppose that the human leukocyte count in the peripheral blood is $10^4/\mu\text{L}$ in which 60% of cells are neutrophils and the blood volume is 5 L, the absolute number of neutrophils in circulation is 3×10^{10} . This is only 1–2% of the total number of neutrophils in the whole body in physiological conditions. One of the unique features of neutrophils is that those in the order of 10^{11} (10^7 in mice) are disposed every day after a rather short life span of 12 h [4]. An antiapoptotic BCL-2 family member Mcl-1 appears to regulate spontaneous apoptosis in neutrophils but not macrophages. Myeloid-specific conditional deletion of Mcl-1 resulted in a selective neutrophil deficiency [5]. Mcl-1 interacts with proapoptotic proteins, such as BID (see Fig. 10.2 in Chap. 10). Different from a single hormone that also has its own life span, depending on the mode by which neutrophils undergo cell death, a variety of cell surface molecules are activated and intracellular molecules are released after the cell death.

Neutrophils that circulate in the longest period of time, therefore aged, are shown to up-regulate CXCR4 expression and down-regulate L-selectin (also called CD62L) expression. CXCL12 expression in bone marrow cells (see Chap. 11, HSC)

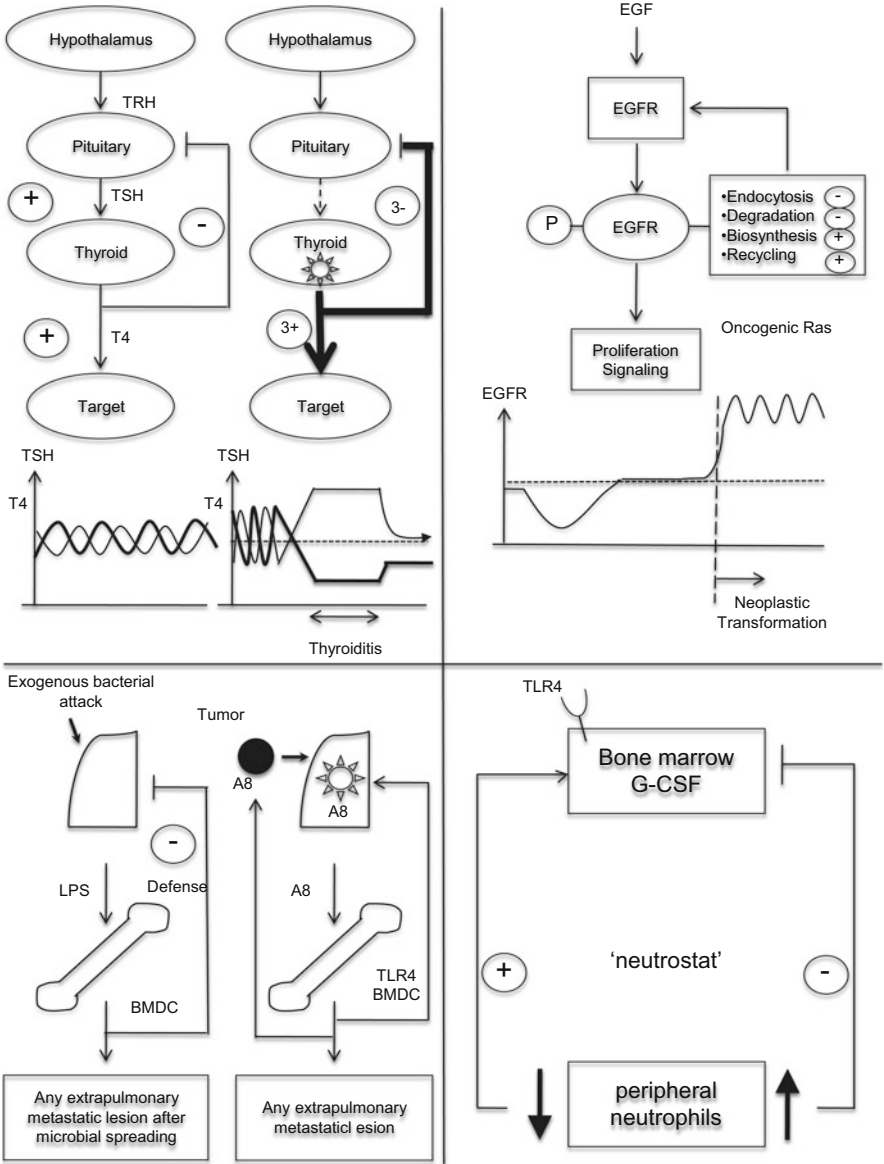


Fig. 14.1 Homeostasis at different levels

serves as a ligand for CXCR4-expressing cells to home back to the bone marrow. Selectin-counter-receptor system-mediated contacts between leukocytes and endothelial cells play a crucial role of transendothelial migration of leukocytes (see Fig. 1.2 in Chap. 1). Therefore, Ly6G⁺CXCR4⁺CD62L^{low} neutrophils expanded in

circulation by the failure of homing back in knockout mice of endothelial selections (E-selectin and P-selectin) or fucosyl-transferase [6].

Multiphoton microscopic analysis revealed that injected DsRed-labeled aged neutrophils displayed dynamic and frequent contact with resident macrophages in the bone marrow that expressed the liver X receptors α and β (LXR), an oxysterol-activated transcription factors. During clearance of the aged neutrophils by the macrophages, LXR-dependent down-regulation of CXCL12 took place as evidenced by the synthetic LXR agonist GW3965 and double knockout mice of LXR α and β . Transfusion of bone marrow-derived neutrophils that became aged *ex vivo* into mice at 10^6 cells per mice that is comparable to that in physiological clearance induced reduction of CXCL12 proteins and 2.4-fold increase in CFU-C and LSK cells in circulation that play both regenerative and immunological roles in tissues, whereas numbers of mature leukocytes and G-CSF levels were not changed. Myeloid-specific deletion of CXCR4 failed to induce neutrophil homing back to bone marrow and conditional deletion of CXCL12 from stromal cells promoted release of progenitor cells. Thus, neutrophil disposal in bone marrow appears to control HSC niches via the CXCR4-CXCL12 axis. Although molecular mechanisms responsible for LXR activation remain to be elucidated, endogenous cellular factors derived from the aged and dead cells presumably control the neutrophil homeostasis.

When neutropenia is induced under cytotoxic drugs in clinical settings or immune-depletion by antibodies (Abs) against Ly6G, such as RB6-8C5 or clone 1A8, in experimental conditions, cell deaths should take place to exert low levels of inflammation. The Ab-induced neutropenia promoted expansion of LSK, CMP, and GMP (see Fig. 2.1 in Chap. 2, refer to Chap. 11 for LSK), which was dependent on G-CSF. Given that the response could occur in NSG mice, it is independent of T cells, B cells, and NK cells. Plasma G-CSF levels were unchanged when mice were subjected to granulocyte transfusion, but G-CSF mRNAs were reduced in G-CSF-treated mice with leukocytosis. G-CSF-KO and G-CSFR-KO mice exhibited neutropenia in physiological conditions. Given that increases in plasma G-CSF levels and LSK and GMP populations in Ab-induced neutropenia could occur even in germ-free mice, those positive feedbacks are unlikely to be due to indirect effects by infection induced by the neutropenia. However, those feedbacks were abrogated in knockout conditions in either TLR4 or TRIF but not MyD88 [7]. As the possible sensor in <neurostat,> as a metaphor to thermostat, the authors assumed TLR4 in bone marrow stromal cells that may secrete G-CSF toward HSC niche (Fig. 14.1).

G-CSFR-KO mice crossed with CD68:mGCSFR mice, in which G-CSFR is expressed in a monocyte-specific manner under the control of macrosialin (CD68), were neutropenic due to the loss of G-CSFR in granulocytic cells, but G-CSF-induced mobilization of both LSK cells and CFU-C were normal [8]. I suppose that endogenous TLR4 ligands, such as S100A8, enriched in neutrophils might play a regulatory role in the neurostat.

This assumption is supported by the results of experiments of <emergency granulopoiesis> induced by LPS, in which mice that underwent reciprocal BMT between wild-type and TLR4-KO mice were challenged with LPS and both the

plasma G-CSF levels and CD11b⁺Gr1⁺ cell counts in the peripheral blood failed to increase in mice whose stromal cells in bone marrow were TLR4-KO [9].

14.2.2 Organ Level

14.2.2.1 Heart

Fibroblasts are more abundant than cardiomyocytes in the heart, and the reciprocal interaction between the two types of cells plays a protective role against hemodynamic overload in a homeostatic manner [10, 11]. Mechanical stretch induces secretion of angiotensin II (ATII) in cardiomyocytes, which binds ATII type A1 receptor (AT1A) in fibroblasts to stimulate their proliferation and production of growth factors, such as IGF-1 in a KLF5-dependent manner. IGF-1 then induces hypertrophy of cardiomyocytes, thus protecting against pressure overload, blockade of which by a peptide inhibitor of IGF-1 aggravated heart failure shifting from a compensatory to decompensatory state (see Fig. 15.1 in Chap. 15).

14.2.2.2 Liver

There is a reciprocal stimulation loop between hepatocytes and sinusoidal endothelial cells (SEC) in liver. Hepatocytes produce VEGF, which binds and stimulates VEGFR2 in the SEC. SEC, in turn, secretes HGF to stimulate hepatocytes. The bidirectional signaling supports the survival and maintenance of hepatic architecture.

14.2.2.3 Adipose Tissue

Homeostatic sensing mechanisms appear to exist between adipocytes and adipose macrophages. More than 90% of macrophages in white adipose tissue in obese db/db mice and human fat biopsy samples from obese patients surround necrotic, but not apoptotic, adipocytes, which are recognized as crown-like structure by optical microscope. A positive correlation has been reported between frequency of dead adipocytes versus adipocyte size in the same samples as well as those from mice deficient in hormone-sensitive lipase (HSL) that lack increased adipose mass or obesity [12]. Hypertrophied and normal adipocytes produce saturated fatty acids (SFA), such as palmitate <16:0> (see below), which binds and activates TLR4 in macrophages to secrete TNF α . TNF α induces programmed necrotic cell death in adipocytes, which lacks chromatin condensation and apoptotic bodies commonly seen in apoptosis. Conversely, the necrotic adipocyte death is not accompanied by gross neutrophil infiltration as observed in standard necrosis. It is likely the adipose macrophages protect against the release of intracellular substances that usually

exert inflammation, such as neutrophil infiltration. The counteraction against the expansion of fat mass regulates the adipose tissue homeostasis.

This is similar to sterile inflammation in Guillain-Barré syndrome as stated in Chap. 4 in which macrophages are found to scavenge the damaged axons. In dopaminergic neurons, both synthesis of dopamine from tyrosine by tyrosine hydroxylase and dopamine oxidation by monoamine oxidase are accompanied by production of ROS. The ROS needs to be scavenged by antioxidant systems in a homeostatic manner, disruption of which results in apoptosis or, if ROS is of high level, necrosis in the neuron in Parkinson's disease [13].

14.2.3 Cell Level

14.2.3.1 EGFR

Let us think of homeostasis at a cellular level. EGFR is expressed in a variety of cells, such as epithelial cells and fibroblasts. Although they can proliferate in vitro only in the presence of fetal serum without EGF, EGF promotes their growth. If you have cell number as an effect of EGF, it just increases and never remains constant upon EGF stimulation, and therefore homeostasis is beyond discussion. However, if you have EGFR number on the cell surface as an effect of EGF, you can see that it is under homeostatic control [14]. Upon EGF stimulation of NIH3T3 fibroblasts stably expressing EGFR-GFP, rapid activation of the receptor as monitored by autophosphorylation starts in 2 min, peaks between 2 and 10 min, and lasts up to 60 min. The number of EGFR-GFP on the cell surface as judged by ^{125}I -EGF binding begins to decrease in 15–30 min by endocytosis leaving 25% of the receptor in 2 h [15]. But actual internalization can be kinetically detected in 1–5 min. The endocytosed receptor is eventually subjected to degradation by proteasomes. Generally the half-life of EGFR is 8–24 h, but it dramatically decreases to 90 min upon EGF stimulation as measured by ^{35}S -labelling experiments.

Roughly 20% of the endocytosed receptor in early endosomes is recycled to the cell surface and EGF stimulates growth-promoting signals to up-regulate biosynthesis of the receptor [16]. Here you can see negative regulation by endocytosis and degradation and positive by recycling and new biosynthesis (Fig. 14.1). The cell surface receptor ultimately returns to the state before stimulation in both number and enzymatic state in 6 h, which is followed by cell division with doubling times ranging from 12 to 24 h.

While the homeostatic balance is perturbed in a long period of time in thyroiditis by acute release of T4 from damaged T4-producing cells, disturbance of EGFR on the cell surface also has been recognized in naturally occurring genetically mutated EGFR in lung and brain tumors. The EGFRvIII mutant lacking the sequence responsible for ligand binding and dimerization is constitutively autophosphorylated but has impaired

endocytosis and degradation, during which period of time its sustained activation transforms the cell [17].

14.2.3.2 CXCR2

Polymorphonuclear leukocytes (PMN) as represented by neutrophils migrate to the inflammatory sites to kill bacteria in a chemokine and its receptor-dependent manner. One such system is the MIP-2-CXCR2 axis critically transduced by PI3K γ , which not only stimulates cell migration but also gives negative feedback on it by up-regulating the expression of GRK2 that desensitizes the axis by promoting CXCR2 internalization, i.e., reducing the CXCR2 number in the cell surface. By this homeostatic control, tissue destruction by, for example, too much PMN-derived ROS may be avoided. When the level of bacterial assault is large, the negative feedback needs to be shut off to ensure the persistent mobilization of PMN. This is executed by TLR4 signaling-activated MEKK that inhibits GRK2 [18].

14.2.4 *Molecular Level*

14.2.4.1 NF κ B Activation

Before explaining homeostatic regulation of NF κ B, I briefly will review the interaction between c-fos and NF κ B, because those two transcription factors are critically involved in an inflammatory tumor microenvironment as exemplified by transcription of VEGF mRNAs. NF κ B activation results in VEGF expression, and down-regulation of NF κ B leads to diminished expression of VEGF. AP-1, including c-fos, is transcriptionally activated by serum response factor (SRF) and ternary complex factor (TCF), and c-fos is potent among other AP-1 species in VEGF expression [19]. NF κ B is activated by degradation of an inhibitory NF κ B-binding regulator I κ B. NF κ B induces expression of TCF, such as Elk-1, which activates c-fos transcription and subsequent VEGF expression in pancreatic tumor cells. In short, NF κ B activates c-fos through Elk-1. In morphine-induced activation of NF κ B and c-fos in neuronal cells, both are responsible for transcription of I κ B to levels high enough to inhibit NF κ B. Thus, c-fos inhibits NF κ B through I κ B. Therefore, in actual tumor microenvironment, it is likely that constant tumorigenic c-fos and inflammatory NF κ B signals are out of control in the negative feedbacks.

14.2.4.2 NF κ B Modulators

NF κ B is the most representative molecule of inflammation that is known to be both positively and negatively regulated during activation. One way to help

understanding brakes in the NF κ B signaling is to see what would happen when elements involved in the negative feedback, A20 and miR-146a, are genetically knocked out in mice. Targeted disruption in mice of NF κ B p50 subunit by itself revealed no developmental activity but displayed susceptibility to infection by *Streptococcus pneumoniae* with B cells not growing in response to LPS [20]. I remind you that the two hallmarks of inflammation are mobilization of white blood cells and vascular permeability. You can never discuss inflammation with, for example, just IL-6 expression at cell levels. Readers should bear in mind the fundamental information on NF κ B signaling as described in Chap. 2.

A20 Ubiquitination

A20, also known as TNF α -induced protein 3 (TNFAIP3), is an ubiquitin-editing enzyme of dual function containing both N-terminal deubiquitinating and C-terminal E3 ligase domains [21]. TNF α induces A20 expression ubiquitously, which in turn gives negative feedback loops.

In the TNFR activation cascade, E3 ubiquitin ligases cIAP-1 and -2 (cellular inhibitor of apoptosis proteins) polyubiquitinate RIP1 (receptor interacting protein). A20 deubiquitinates K63-linked polyubiquitin chains from RIP1 and instead ubiquitinates it to form K48-linked chains [22]. This ubiquitination switch abrogates RIP1 binding NEMO and RIP1 is subjected to degradation by proteasome. TNF α is induced by a variety of stimuli, exogenous and endogenous, and TNF α -stimulated NF κ B activation is limited this way by A20 that is simultaneously induced to avoid expanded NF κ B signaling. A20 modifies the ubiquitination status of RIP1 for proteasomal degradation in TNF α R signaling. In TLR4 signaling, A20 works in concert with the regulator TAX1BP1 to disrupt interactions among the E3 ligases, TRAF6, TRAF2, and cIAP1, and the E2 enzymes, Ubc13 and UbcH5c. Both of the E2 enzymes are ubiquitinated for proteasomal degradation, which can be blocked by A20-deficiency. As expected, A20-KO mice fail to terminate inflammation, resulting in severe inflammation in multiple organs, susceptibility to infection, and cachexia at age 3–6 weeks after birth due to lack of negative feedbacks [23]. Birth under Mendelian ratios shows lack of the effect on embryogenesis, but A20-KO mice were runts and started to die a few weeks after birth. Spontaneous occurrence of inflammation in multiple organs, including hepatitis and colitis accompanied by cryptic abscess, with infiltration of Mac1⁺Gr1⁺ granulocytes and Mac1⁺ macrophages, which was not affected by doubly KO of RAG1, strongly suggests homeostatic roles of A20 in innate immunity with activated lymphocytes being dispensable for the lethal inflammation.

Induction of inflammation on purpose by endotoxin further supports the inhibitory property of A20. All A20-KO mice were documented to be dead within 2 h of LPS injection at 5 mg/kg, whereas A20^{+/−} hetero mice survived even at 25 mg/kg. Pulmonary inflammation takes place in a stochastic manner (Dr. Averil Ma, personal communication), which cannot be rescued by TNF α -KO.

Myeloid cell-specific deficiency of A20 in mice up-regulated serum levels of TNF α , IL-1 β , IL-6, and MCP-1 (CCL2) [24]. A20 is expressed in HSC. Hematopoietic cell-specific knockout of A20 in mice revealed a decrease in LT-HSC pool (see Chap. 11), loss of their quiescence, and reduced expression of p57 [25].

A genome-wide association study (GWAS) with British population identified A20 as a Crohn's disease susceptible gene (see Chap. 8).

miR-146a

miR-146a negatively regulates 2 NF κ B signaling molecules TRAF6 and IRAK1 [26, 27]. Chronic and low-grade inflammation that develops without any stimuli on purpose during the course of aging was reported in miR-146a-KO mice. The inflammation is aggravated in response to stimuli, such as endotoxin. Lack of brake for quiescence in hematopoietic stem cells (HSCs) in miR-146a-KO mice resulted in myeloproliferation and ultimately exhaustion of long-term HSCs and LSK cells [28]. The phenotypes were partially rescued by double KO with RAG1 and almost perfectly with IL-6. The alteration in HSCs is not cell-autonomous, because IL-6 derived from T cells by the same mechanism of NF κ B deinhibition by miR-146a-KO stimulates HSCs to massive proliferation of blood cells.

Of note, by utilizing transgenic NF κ B-GFP reporter mice in which RelA loading on the *cis*-NF κ B enhanced GFP transgene promoter and on those of NF κ B-responsive genes, including CCL2 and IL-6 as judged by ChIP analysis, approximately 6% and 16% of LSK and HSCs were found to have basal levels of NF κ B activity at ages 2 and 8 months, respectively, which increased to 26% at 6 h after LPS stimulation with 2-month-old mice. In 146a-KO mice bred with NF κ B-GFP reporter mice, GFP-positive LSK cells and HSCs scored more than 20% at age 2 months [28].

What causes the noise levels of NF κ B activity in the wild-type mice? I suppose it is the homeostatic levels of inflammatory activity as monitored by the NF κ B reporter.

14.3 Sterile Inflammation

Microbe-induced inflammation contrasts with sterile inflammation that takes place in the absence of microbes.

14.3.1 Neurogenic Inflammation

Although the idea of neurogenic inflammation, in a sterile sense, was originally proposed in 1910 by A. N. Bruce who showed a sensory nerve dependency of

mustard oil-induced inflammation that accompanied vasodilatation [29]. Currently, it has been shown that cooperative activation of TRPA1 and TRPV1 by mustard oil contributes to the behavioral avoidance to the chemical and vascular permeability is increased by neuropeptides, such as substance P (SP), which activates release of histamine from mast cells that were observed to be directly associated with SP-expressing neurons. This inflammation is nonmicrobial and sterile inflammation caused by an exogenous ligand. A well-known, clinical disorder migraine is mediated by neurogenic inflammation with meningeal vasodilatation around meningeal sensory neurons involving SP, histamine and serotonin, and histamine/serotonin receptor antagonists. Precise triggering mechanisms of migraine are still unknown; general stress may be engaged. Immobilization stress experiments in rat induced dura mater mast cell degranulation. Thus, migraine is sterile inflammation elicited by endogenous ligands.

14.3.2 *Levels of Inflammation*

The discrimination is based on the source of stimuli, whether exogenous or endogenous, without considering the levels of inflammation: the higher the levels, the more pathological or even life-threatening. Acute inflammation is eventually resolved. Cell dynamics and tissue remodeling in both the inflammatory and subsequent resolution phase are described at tissue levels in Chap. 1.

For example, bacterial infection causes tissue destruction (inflammatory phase), which is followed by the tissue regeneration with elimination of bacteria (resolution phase). If the levels of inflammation are high as exemplified in sepsis, the inflammation induces disease. However, if they are as low as physiological levels, the individual is seemingly healthy. In addition, this physiological inflammation can be caused by stimuli of endogenous origin. This is what I call homeostatic inflammation (see below for an example of club cells) (Fig. 14.2).

Concepts similar to homeostatic inflammation have been proposed, such as para-inflammation [30], senescence-associated secretory phenotype (SASP) (see Chap. 10), and senescence-associated inflammatory response (SIR) [31] (Fig. 14.3).

Under tissue stress without evident injury, an adaptive process, para-inflammation, is induced not only to cope with the noxious circumstances but also to restore the status to physiological. Therefore, para-inflammation is low-grade inflammation whose process could be reversible. If maladapted, the tissue undergoes conversion to pathological settings with chronic inflammation, as exemplified in type 2 diabetes and atherosclerosis. Importantly, while molecular pictures of the overt disease state have been focused and studied extensively, precise understanding of the para-inflammatory stage lacked physical and mechanistic examples.

SIR was proposed in mice deficient in casein kinase 1 α (CK1 α), which negatively regulates Wnt signaling by inducing β -catenin degradation through phosphorylation in concert with GSK3 β [35]. There is a small overlap in the gene

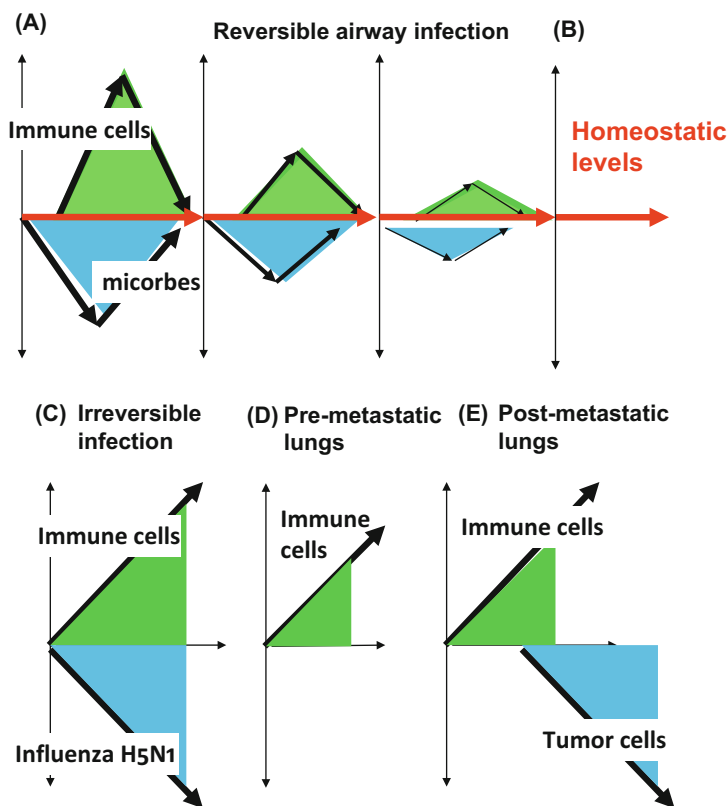


Fig. 14.2 Consequence of inflammation

Images of various levels of inflammatory responses against microbes are depicted schematically (A). The levels can be as low as homeostatic levels (B). A lethal respiratory infection, such as H5N1, divergence instead of convergence is assumed (C). Premetastatic lungs are recruited with immune cells but devoid of microbes and tumor cells (D). After arrival of tumor cells, both tumor cells and immune cells expand (E)

signatures between SASP and SIR [36]. Ablation of CK1 α resulted in activated Wnt signaling in colon hyperplasia without tumorigenesis [37]. p53 was activated to counteract tumor formation, and this was accompanied by sustained DNA damage response (DDR) (see Chap. 10). Simultaneous inactivation of p53 allowed tumor formation. Colon SIR showed the senescence marker SA- β -gal but lacked infiltrating leukocytes according to the claim of the authors, indicating that SIR is of cell-autonomous. In this regard, SIR does not conform well to the classical definition of inflammation but can be an extension of para-inflammation. However, both lungs and colon serve as a barrier against exogenous microbes and chemicals and therefore constantly station leukocytes mobilized through circulation. Homeostatic inflammation includes the homeostatic systems by which to recruit the physiological levels of immune cells. Both mPGES and PLA2 were highly induced in colon

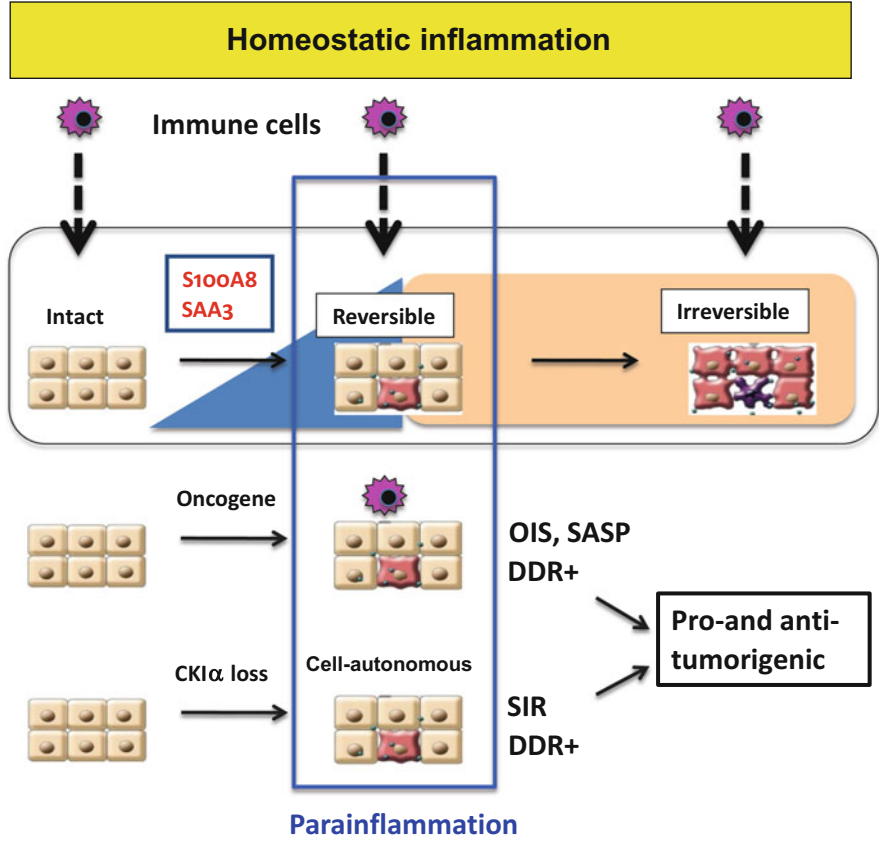


Fig. 14.3 Concepts of inflammation
In addition to lung premetastasis [32], the idea of homeostatic inflammation can be extended to obesity and responses to nucleic acids [33, 34]. *DDR* DNA damage response

SIR, and the process was reversible by NSAID. This contrasts with SASP as exemplified in oncogene-induced senescence (OIS), in which cytokines, such as IL-6 and IL-8 and their corresponding receptors, are dominantly induced through NFκB and C/EBPβ to recruit immune cells and are necessary and sufficient to maintain OIS in a non-cell-autonomous fashion [38, 39].

14.3.3 Interface between Microbe (Non-self) and Host (Self)

Skin, intestinal tract, and respiratory tract are the interfaces between host as self and the external world as non-self, including bacteria producing LPS. The total surface area of epithelial barrier in the gastrointestinal tract (GIT) in humans is approximately 200 m² carrying 10¹⁴ bacterial inhabitants and that of alveolar space is

160 m² normally without microbes. Dermal surface area is roughly 2 m² with 10¹² bacteria usually lacking Gram-negative ones. Thus in contrast to GIT and skin that are always stimulated by bacteria, airways are constantly exposed to assaults of airborne exogenous ligands, including microbes and chemicals. In any cases, host defense mechanisms are required in those interfaces in a homeostatic manner.

Skin is an unfavorable environment for bacterial growth, but antimicrobial peptides, such as BPI (see Chap. 5), are known to play a protective role in the skin.

14.3.3.1 Gastrointestinal Tract

Although molecular mechanisms are still unknown, mice supplemented by drinking water with low-molecular weight dextran sodium sulfate (DSS) exhibit colitis, i.e., damages in epithelial cells and up-regulation of cytokines, such as TNF α , IL-6, and IFN γ . MyD88-KO, TLR4-KO, or TLR2-KO mice were more sensitive to DSS displaying colon bleeding [40]. Depletion of commensal bacteria by antibiotics before DSS failed to affect mortality of the animal. Colon bleeding occurred at 3 days before leukocyte infiltration at 5 days after DSS in MyD88-KO mice. Neither bacterial dissemination in the spleen nor increased leukocyte infiltration in the colon was found. The sensitivity to colitis also was induced by irradiation. Those mice displayed reduced expression of IL-6 and KC that are thought to be involved in the repair process. Interestingly, supplementation of purified LPS or LTA in drinking water, which mimics commensal bacteria, prior to DSS in microflora-depleted mice by antibiotics rescued the DSS-induced colitis. This experiment clearly indicates the role of TLR4 in the protection against the external world in a homeostatic manner.

14.3.3.2 Lungs

Lungs are a frequent target of metastasis.

Alveolar Space

Alveolar space is basically sterile in normal conditions in which macrophages predominate over neutrophils. Intratracheal instillation of LPS in mice induced recruitment in bronchoalveolar lavage (BAL) of neutrophils and monocytes that peaked at 24–48 h and 48–72 h, respectively, posttreatment [41]. It is likely that bacteria-derived LPS in the airway activates TLR4 in macrophages and epithelial cells in the airway (see Chap. 2), which produces TNF α and IL-1 β . Those cytokines in turn stimulate macrophages and epithelial cells to secrete the CXC subfamily chemokines, such as CXCL1 (GRO α), CXCL2 (GRO β), and CXCL8 (= IL-8, murine equivalent is called KC) and the CC subfamily ones as represented by CCL2 (MCP-1). Knockout mice of CXCR2, which is a common receptor for the

above-mentioned CXC chemokines, displayed defective recruitment of neutrophils in response to LPS [42]. CCR2-KO mice, which can hardly signal from CCL2, also exhibited dramatically reduced alveolar recruitment of monocytes. In LPS-challenged CCR2-KO mice, CCL2 was elevated in the BAL. This reminds us of the negative feedback by thyroid hormones for TSH. Lower levels of T4 correspond to impaired monocyte mobilization due to CCR2-KO, which increases TSH levels and CCL2 concentration in BAL, respectively. Conversely, given that T4 can down-regulate TSH levels, it is expected that CCR2⁺ monocytes may decrease CCL2 in the BAL. In fact CCL2 was consumed or decreased in a time-dependent manner by CCR2⁺ monocytes but not neutrophils. Importantly, in both cases of thyroid and BAL, readers can now understand the homeostatic controls by endocrine factors and/or cells and that monocyte recruitment in BAL is an inflammatory process, although physiological levels of stimulation by those chemokines can manifest homeostatic inflammation. However, CCL2 expression is regulated by many ways as stated in Chap. 2, i.e., positively by CRP and negatively by PGE2.

Club Cells

Club cells, previously called Clara cells, are specific resident epithelial cells in the terminal bronchioles that play a xenobiotic role against airborne pathogens and pollutants. Renaming was proposed by experts of international respiratory societies because the eponymous German anatomist, Max Clara, was an active Nazi who used tissues from executed prisoners [43]. For example, naphthalene induces cell death of club cells by naphthalene metabolites 2',5'- epoxide synthesized by CYP2F2 that is specifically expressed in club cells. Both mouse and human club cells produce club cell secretory protein (CCSP) that is thought to have anti-inflammatory roles. Roughly 2% of bone marrow cells are CCSP-positive. Naphthalene treatment increased CD11b⁺CD45⁺CYP2F2⁺CCSP⁺ cells in both the peripheral blood and bone marrow 2 h through 20 days [44]. When the same experiment was performed with CCSP-KO mice that underwent BMT with GFP⁺CCSP⁺Sca-1⁺ cells from GFP transgenic mice, GFP⁺CCSP⁺K18⁺ cells of mature epithelial morphology could be observed in the lungs. Given the mobilization of club cell progenitors from bone marrow to lungs in the acute lung injury by naphthalene, genetic lung disorders with lung epithelial destruction, such as cystic fibrosis (CF), may involve disturbance of club cell dynamics. In fact, it has been reported that CF patients have increased numbers of CCSP⁺ cells in both the peripheral blood and bone marrow [45]. I suppose that even under physiological conditions CCSP⁺ cells constantly reconstitute the airways with low levels of damages in a homeostatic manner. The CCSP⁺ cells express chemokine receptors, such as CCR2 and CXCR4, and migrate in response to SDF-1. In addition, S100A8 expression was up-regulated in CF mouse model with G551D mutation in CFTR (CF transmembrane conductance regulator) and more sensitive to LPS than the wild-type mice [46].

How do CFTR mutations cause persistent lung inflammation in CF? At least at the beginning, endogenously provoked inflammation takes place, which persists to eventually allow lung epithelial barrier disruption.

For example, defective CFTRF F508del up-regulated ROS and tissue transglutaminase, which impair autophagy by cross-linking Beclin-1. This results in failed clearance of aggresome as monitored by HDAC6 [47]. CF airway tissues were reported to show ceramide accumulation and NF κ B activation.

Disruption of the club cell barrier may allow entry of small amounts of bacterial components, such as LPS, into circulation, or cellular components derived from dead club cells may sensitize the adjacent club cells still alive, both of which results in club cell secretion of chemokines, including SDF-1 as stated and SAA3, which we have shown was drastically up-regulated by intratracheal administration of LPS.

Club cells express TLR4 [32]. Genetic deficiency in either TLR4 or MyD88 in C57BL/6J mice has been reported to induce pulmonary emphysema in high-aged mice of 3–12 months, in which destruction of airways from terminal bronchioles to alveoli took place in a manner independent of strain since C3H/HeJ and C57BL/10ScNJ also were affected (see Chap. 5) [48]. Mechanistically, TLR4-KO resulted in up-regulation of Nox3 in the lungs, whereas levels of Nox1, Nox2, and Nox4 were unchanged, which dramatically reduced the levels of antioxidant in BAL as judged by bathocuproine-Cu²⁺ assay with uric acid as an antioxidant standard. Although ROS is increased in BAL, which could be inhibited by N-acetyl cysteine (NAC) or Nox inhibitors, such as apocynin and diphenylene iodonium (DPI), the disease progression is slow and they could find increase in neither inflammatory cells nor cytokines, including TNF α , VEGF, and IFN γ .

Repeated LPS exposures twice a week for 5 weeks induced pulmonary emphysema in 6 months. This appears to represent chronic exposure to high levels of LPS in apparent inflammatory response, such as repeated pneumonia. Normally, air contains LPS less than 1.0 EU/m³ (0.44 in Southern California), which may fluctuate in a time- and space-dependent manner [49]. The sensing and protecting roles played by TLR4 is likely to maintain the lung homeostasis.

In contrast to lungs, commensal bacteria always reside in gut mucosa and recognized by TLR4 but under tight control to avoid overinflammatory response. In both cases, TLR4 appears to have a protective role against the external world in a homeostatic manner.

As I stated in Chap. 13, premetastatic lungs have increased vascular permeability compared with the normal lungs of non-tumor-bearing. The low levels of vascular permeability in normal lungs before tumor challenge reflect the physiological levels of inflammation in the lungs, which is included in the concept of homeostatic inflammation (Fig. 14.3) [32] (see also Chap. 15).

14.3.4 *Interface between Transformed Self and Host (Self)*

In contrast to the rather clear interfaces between microbes and host in skin and respiratory tracts, there are those between transformed self and host (self).

14.3.4.1 **Uric Acid**

The final product of purine catabolism by xanthine oxidase (XO) is responsible for converting hypoxanthine and guanine to uric acid (UA). UA causes gout, an inflammatory disease of the articular cavity where its crystals, as transformed self, formed usually due to increased serum levels, activate inflammasome.

What is the physiological function of UA in a homeostatic sense? This issue can be contemplated by two conditions: its overproduction and deficiency. A rare X-linked recessive disorder, Lesch-Nyhan disease, is caused by mutations of hypoxanthine-guanine (H-G) phosphoribosyltransferase (Hprt), which is expressed in almost all types of cells in the body and is essential for recycling HG into purine pools for DNA synthesis [50]. Therefore, the affected male patients suffer from hyperuric acidemia and gout. Selective loss of dopamine by an unknown mechanism in more than 50% in the basal ganglia during development is thought to contribute to motor disturbance and behavioral problems, such as self-mutilation. Hprt-KO mice almost phenocopy the symptoms of Lesch-Nyhan disease.

Conversely, XO-KO mice were runted and failed to survive beyond 6 weeks after birth due to maldevelopment. The serum UA in XO+/- mice was reduced by roughly 50% as expected [51]. XO+/- female mice displayed epithelial collapse and tissue necrosis of mammary glands. Different from patients with XO mutations, the lactation problem took place in XO+/- mice, and their pups were unable to live beyond 12 days postpartum. The primary reason is that XO is necessary for enveloping milk fat droplet. Intriguingly, XO+/- mice became obese with aging with inflammation in the adipose tissue, which revealed increased number of F4/80⁺ macrophages, up-regulation of CCL2 and TNF α , and activation of PPAR γ [52].

The information cannot establish any roles of UA in physiological settings. To complicate the story, XO uses molecular oxygen O₂ as an electron acceptor to generate ROS. Therefore, deficiency of UA simultaneously is assumed to accompany less production of ROS in XO+/- mice in sites where XO is the major critical source of ROS. However, it safely can be said that UA levels are maintained in a homeostatic manner and its overproduction alarms the host by painful gouty inflammation. The event that underlies its deficiency is life-threatening and also induces inflammation.

14.3.4.2 Adipose Tissue (Low-Grade Inflammation)

Saturated fatty acid (SFA) from hypertrophied adipocytes is the transformed self in terms of its high quantity but not its quality change. Obesity by itself does not cause pain as in the case of gout to alarm the host of danger. However, abundant SFA stimulates TLR4 in the macrophages in the adipose tissue, which in turn secrete TNF α as described earlier (see Table 7.1 in Chap. 7 and Fig. 15.1 in Chap. 15). The vicious cycle between SFA from hypertrophied adipocytes and TNF α in the accumulating macrophages induces adipose tissue remodeling in obesity. A mixture of palmitate (hexadecanoic acid) <16:0> and oleate <18:1 omega-9> (see Note in Chap. 16) at 200–400 μ M could activate NF κ B to secrete both IL-6 and TNF α in a manner dependent on the TLR4 expression and TLR4-KO partially protected against insulin resistance induced by a high-fat diet [53]. EPA <20:5 omega-3> inhibited the binding between biotinylated LPS and an N-terminally flag-tagged soluble form of TLR4 that was fused to a soluble form of MD-2 by a flexible linker in a dose-dependent manner [53, 54]. Monocyte recruitment and subsequent conversion and accumulation in adipose tissue of obesity are driven by CCL2, SAA3, and hyaluronan, which are produced by the adipocytes. Activation of the corresponding receptors, CCR2, TLR4, and CD44, respectively, is responsible for the mobilization.

At the level of macrophage, LPS-stimulated NF κ B-mediated activation of transcription was shown to be inhibited by the transcription factor Liver X receptor (LXR). Conversely, lipid A could suppress the LXR agonist-stimulated, LXR-mediated transcription of ABCA1 transporter engaged in cholesterol efflux [55]. The signaling molecule IRF3 physically interacts with LXR.

CD4⁺Foxp3⁺ Treg cells are abundant in the abdominal adipose tissue of mice and participate in the chronic and low-grade inflammation there [56]. They expressed a variety of molecules responsible for leukocyte recruitment, including CCR2 and CXCL2. Mice that expressed diphtheria toxin (DT) receptor under the control of Foxp3 regulatory elements underwent rapid systemic depletion of Treg with DT. In those mice, inflammatory mediators were induced, including TNF α , IL-6, RANTES, and SAA3 in the visceral adipose tissues, and they exhibited insulin resistance. In obese human samples, the ratio of Foxp3/CD3 indicative of Treg in omental fat is significantly lower than that of subcutaneous fat, and correlation between BMI and low Treg was observed.

General Thinking on Molecular Interactions

I highlight molecular brakes in the inflammatory phase. In order to be followed by the resolution phase, the molecular activation needs to be inhibited during the process.

For the sake of simplicity, let us consider just three molecules: A, B, and C.

(continued)

If A activates B and B activates A, the activation status of both A and B should be expanding. In this setting, if A is down-regulated, the activation status of B also is affected to be down, which in turn down-regulates the activation status of A. This is a mutual activation.

If the relationship is a mutual inhibition between A and B, down-regulation of the activation status of A weakens its inhibitory effect on B, which results in augmentation of the activation status of B. The more activated B can give more inhibitory effects on A, leading to further down-regulation.

If A activates both B and C, and C gives negative feedback on A at the same time, the A to B positive signaling never expands endlessly. If C gives a positive feedback, the situation between A and C is the same as the mutual activation. The unidirectionally activated B by A also fails to subside.

14.4 Chronic versus Repeated Acute Inflammation

Persistent presence of stimuli that cause inflammation induces chronic inflammation. For example, if host can totally eliminate exogenous microbes inducing acute infection, but the infection occurs repeatedly with significant length of intervals in between, this cannot be called chronic inflammation. However, if the interval is too short to give the cell, tissue, or whole body normal context, the situation may be chronic inflammation.

Homeostatic inflammation is thought to be chronic or at least repeated acute inflammation of physiological levels.

14.5 Homeostatic Stabilization Controls (Neuronal Sensor vs. LPS)

Sensor system accepts a variety of signals of both extrinsic and intrinsic origin before appropriate responses at organ or individual levels. To cope with the multiple signals, one way is to put regulatory elements between the acceptor and effector system. A classical example is found in the nervous system: sensory neurons accept signals, such as heat or pain, which are transmitted to the central nervous system (CNS). The efferent motor neurons reach the skeletal muscle for suitable movement to escape from the heat- or pain-generating target. We know that there are spinal cord interneurons that inhibit the efferent neurons. The nociceptive interneurons also can inhibit pain-signaling afferent neurons to relieve pain. This is augmented by morphine. Thus, the logics in the reflex are that acceptor signals to CNS, which in turn casts effector signals that are modulated by intervening signals.

By analogy to the motor reflex, <inflammatory reflex> has been proposed by Kevin J. Tracey [57]. The idea involves both neurons and immune cells. An interesting observation was that up-regulation of TNF α after an LPS exposure in 60–90 min was inhibited by vagal (cholinergic) or postganglionic splenic (adren-ergic) nerve stimulation. It was reported that 4.4% of CD4⁺ T cells express Ach in response to β 2 adrenergic receptor (AR) activation. Nude mice deficient in T cells fail to suppress TNF α production in response to vagal stimulation, and this can be rescued by T cell reconstitution [58]. Secreted Ach then stimulates α 7 nAChR on macrophages [59]. Human nicotinic Ach receptors are homo- or hetero-pentameric receptors consisting of 16 different subunits [60]. Serum TNF α levels after LPS stimulation were twofold higher in α 7-KO mice [59]. Adenylate cyclase 6 associated with α 7n AChR [61] activates cAMP-dependent phosphorylation of CREB to up-regulate c-fos, which then inhibits NF κ B responsible for TNF α production. Now readers understand the presence of effector neurons negatively regulating TLR4-activated macrophages. The corresponding afferent or sensory neurons expressing TLR4 for LPS is the vagal nerve, because vagotomy abolished LPS-induced pyrogenic responses. The assumed LPS-induced inflammatory reflex is that the acceptor vagus signals to CNS, which casts the efferent vagus that ultimately culminates in intervening T cells regulating LPS-stimulated macrophages. The purpose of the negative regulation by efferent neurons is presumed to avoid overresponses in LPS-activated macrophages.

In the absence of LPS, the homeostatic stabilization needs to be accomplished by physiological levels of endogenous TLR4 ligands working in place of LPS.

14.6 Disturbance of Homeostatic Inflammation

Oncogenic Src activation in immortalized mammary cells activates both NF κ B and miR suppressor LIN-28B. Whereas NF κ B activates transcription of IL-6, LIN-28B suppresses Let-7 that is capable of inhibiting IL-6 expression via its 3'UTR [62]. Both the single-positive and double-negative signaling cascades up-regulate IL-6 expression. In addition, the up-regulated IL-6 activates NF κ B as a positive feedback. Therefore, once oncogenic Src is activated, amplification of activated NF κ B and IL-6 signaling cascades leads to cell transformation and mammosphere formation with additional expression of VEGF-A, IL-1 β , and IL-8. This explains proinflammatory nature of tumor cells, because the secreted cytokines influence the surrounding cells to induce mobilization of leukocytes and vascular permeability in endothelial cells.

For example, angiogenesis is controlled both positively and negatively as I stated in Chap. 11, imbalance of which gives pathological circumstances the host. Homeostatic inflammation includes homeostatic vascular permeability that allows entry of homeostatically mobilized cells into the sites of inflammation. Bi-potent factors for <permeability and chemotaxis> are candidate inducers for homeostatic inflammation. Let us take VEGF for an example.

The Orf virus of poxvirus family, responsible for contagious pustular dermatitis with high vascularity, edema, and leukocyte infiltration in sheep and humans, carries VEGF-E in its genome [63]. VEGF-E signals through VEGFR2 in host endothelial cells. Although the VEGF-E gene was presumably acquired from the mammalian genome during evolution of the virus, and many other viruses do not have VEGF homologs, we find one extreme of homeostatic inflammation here. VEGF-A of endogenous origin controls vascular homeostasis, as I stated in many parts of this book. The exogenous VEGF-E finds its receptor in host endothelial cells of the limited lesions of infection where the virions are detected only in keratinocytes. If people had found VEGF-E and its receptor VEGFR2 before VEGF-A, we would have been forced to think that host cells have a receptor called VEGFR2 for a specific viral protein VEGF-E with angiogenic activity and VEGFR2 is involved in vascular homeostasis with endogenous ligand VEGF-A.

LPS finds its receptor TLR4 not only in endothelial cells but also various other types of cells in the whole body. Endogenous TLR4 ligands, such as decorin, HMGB1, and S100A8, should control the biology of TLR4-expressing cells in a homeostatic fashion.

Resemblance is found between LPS- and endogenous ligands-induced lung morphology and pathophysiology. The classical experiments by Meyrick and Brigham showed that endotoxemia in sheep induced sequestration of granulocytes in lung microvessels in 15 min, which is followed by interstitial edema and extravasation of those leukocytes across endothelial barrier [64]. The endothelial cells are of electron dense with increased number of pinocytic vesicles, suggesting that endothelial integrity is breached. This mimics vascular permeability lesions that are provoked by S100A8 and SAA3.

In intranasally infected BALB/c mice with influenza virus (H2N2), S100A8-positive mononuclear cells and epithelial cells were observed, particularly in peribronchiolar regions at day 8 when airway-lining structures were damaged. This declined on recovery at day 12. In this acute infection model, virus-stimulated S100A8 expression could either lose up-regulatory stimuli or gain down-regulatory mediators in the resolution phase of influenza virus inflammation. In premetastatic lung where S100A8 expression is sustained, there should be some mechanisms to support its expression in the microenvironment. LPS can induce both S100A8 and IL-10 mRNA expressions, but the former was significantly inhibited in peritoneal macrophages derived from IL-10 knockout mice [65]. This suggests that the concomitant expression of IL-10 could maintain the up-regulated status of S100A8. However, in premetastatic lung, IL-10 expression is not elevated.

14.7 Concluding Remarks

Homeostasis is maintained by endogenous molecules at whole-body, organ, cell, and molecular levels and plays adaptive roles for exogenous stimuli. If it is accompanied by cell migration *in vivo*, it can be called homeostatic inflammation.

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Chapter 15

Explanation of Metastasis by Homeostatic Inflammation

If inflammation caused by either non-self or self molecules can disseminate throughout the body and inflammatory sites actively allow entry of circulating tumor cells and assist their regrowth, then circulating tumor cells metastasize to the sites of inflammation. However, disrupted sites of homeostatic inflammation do not necessarily guarantee metastatic spread and subsequent regrowth.

Before explaining how tumor cells regrow in premetastatic organs with inflammatory properties, there is a fundamental issue of whether or not extrinsic factor-induced inflammation, systemic or localized, facilitates metastasis in the inflammatory lesion.

15.1 Local Stimuli

15.1.1 Irradiation at Primary Sites

Although a rare case of metastasis of lung cancer to a minor traumatic lesion has been reported, mechanical injury seldom allows metastatic growth of tumor, otherwise surgeons lose their job.

Radiation to the thorax or pelvis of tumor-non-bearing normal mice increased serum TGF β 1 levels by twofold for at least 7 days [1]. MMTV/PyMT transgenic model of metastatic mammary tumor showed that thoracic irradiation at 10 Gy by shielding other portions of the body enhanced lung surface metastasis by threefold at 2 weeks after irradiation. This took place in a manner dependent on TGF β 1 in the tumor cells, because conditional depletion of TGF β RII using Cre/Lox technology reversed the effect. TGF β 1, which is induced by irradiation in a tumor-independent manner, is thought to facilitate directly the survival of the tumor cells. In this case, the lungs were not irradiated to cause inflammation but tumor burden by itself

induce premetastatic conditions in the lungs and irradiation-induced serum TGF β 1 activates CTC to reach the lungs.

UV-irradiated epidermal keratinocytes secrete HMGB1, which activates TLR4 to recruit neutrophils in the irradiated skin in which CD11b⁺ Ly6C⁺ Ly6G⁺ cells dominate [2]. In transgenic mouse model of melanoma that overexpressed HGF and an oncogenic CDK4, repeatedly irradiated mice by UV for 6 weeks exhibited some melanoma cells expanding along the endothelial cells, often observed in human melanoma. This angiotropism enhanced lung metastasis, both of which were abrogated in KO mice of either TLR4 or MyD88. Also in this experiment, lungs were not treated by UV, but the generated melanoma by UV prepared premetastatic soil in the lungs.

In both experiments, irradiation of tumor induced host cell responses, which provide favorable conditions for tumor cells to achieve metastasis, such as potentials to intravasate and survive.

15.1.2 Hepatitis

Meta-analysis of 4049 patients who suffered from chronic liver diseases, including fatty liver, chronic hepatitis virus B or C infection, and cirrhosis, revealed significantly lower rates of liver metastasis in colorectal cancer (CRC) [3]. Readers should remember that the organ first encountered by circulating tumor cells from the primary tumor is liver by CRC, lungs by any tumors that do not drain into the portal vein, and any organs by lung cancer. A more focused analysis of chronic viral hepatitis (CVH) B and C, liver metastasis took place in 2.86% (2 of 70 cases) compared with 16.9% (48 of 284) in non-CVH. In C57BL/6 mice with cirrhosis, which was induced by carbon tetrachloride gastrogavage for 2 months, B16F1 melanoma cell injection via the portal vein caused seven- to ninefold increase in frequency of tumor in the liver by histological analysis in cirrhosis over the untreated group [4]. No surface metastatic nodules were found in the lungs. Intravital kinetic analysis showed that the tumor cell movement was slower in the cirrhotic group. Given the presence of regeneration, fiber generation and inflammatory cells in both clinical autopsy and experimental mouse settings, I assume that inflammatory nature in the liver and its matching with tumor cell property may make a difference.

15.1.3 Bacteria or Lipopolysaccharide

Treatment of B16 melanoma cells with lipopolysaccharide (LPS) or lipid A at 1 μ g/ml each for 48 h, which induced CCL2 expression, followed by extensive washing and subcutaneous implantation, reduced the tumor growth compared with untreated B16 cells in both wild-type and TLR4-KO mice. However, LPS promotes tumor

cell migration in vivo [5]. Supportive evidence has been provided by experiments in which direct intratracheal administration of either *Escherichia coli* or LPS before injection of tumor cells, such as B16F10 melanoma or RM-9 cells, through the tail vein enhanced lung metastasis at 14 days after tumor challenge [6]. Tumor cell injection at any time points earlier than 6 h, but not later than 3 days, of LPS was effective for the enhancement. BAL fluid 6 h after LPS had increased amounts of IL-6, G-CSF, KC, SDF-1, and extracellular ubiquitin. The major chemokine receptor of neutrophils in acute lung inflammation is CXCR2, which is activated by MIP-2 and KC (remember that KC is a rodent version of IL-8 in humans). As with TLR4, CXCR2 is expressed in both blood and non-blood cells, both of which contribute to neutrophil accumulation and pulmonary permeability in the lungs. Both ubiquitin and SFD-1 are known to bind CXCR4, and CXCR4 inhibitor AMD3100 blocked the enhancement of metastasis. The reasonable but important point is that they found no metastasis in the extrapulmonary organs. In our laboratory, LPS administration through a catheter only to the left bronchus with microspheres to make sure of the stimulation sites induced vascular permeability at 48 h and accumulation of intravenously injected labeled tumor cells in the left but not right lung. Therefore, a local TLR4 activation of a given pulmonary segment causes a limited inflammatory space to which tumor cells are allowed to metastasize. If the lung TLR4 is activated by an endocrine manner, the whole lungs should be transformed to be premetastatic soil.

15.2 Systemic Stimuli

15.2.1 Arthritis

MMTV-PyV MT mice display no primary mammary tumor at 9 weeks, but during the period of 18–26 weeks tumors with spontaneous metastasis appear. Rheumatoid arthritis mouse model by type II collagen injection was performed intradermally at 9 weeks of MMTV-PyMT mice to induce arthritis in 11–13 weeks of tumor development [7]. The combined systems at early developmental stages of mammary tumor are assumed to see the systemic inflammatory effect at the premetastatic phase. Bone metastasis took place only in the arthritis mice, and more than twofold increase in lung metastasis was observed in the same group. The arthritis induction caused leukocyte infiltration in the lungs, which was enhanced by roughly fourfold in the background of mammary tumor. Elevation of factors in the lungs and circulation was detected, including IL-17, IL-6, and PGE2. Anti-IL-17 antibody and COX-2 inhibitor both reduced the metastasis. The same group demonstrated that arthritis induced mast cell recruitment in the lungs in a tumor-independent manner, but tumor cell engagement enhanced the mast cell activation underpinning the enhanced metastasis [8]. Anti-c-Kit antibody also was effective to

reduce metastasis. Importantly, the detailed experiments tell us that premetastatic soil can be cultivated by inflammation of other organs.

15.2.2 Allergy

The experimental method to induce allergic inflammation in lungs has been established. Intraperitoneal injections of chicken ovalbumin (OVA) followed by repeated aerosol challenges can provoke mucus accumulation in respiratory tracts and airway hyperresponsiveness, serving as an asthma model [9]. The James J. Lee group has demonstrated that CD4⁺ T-cell-dependent recruitment of eosinophils in both bronchoalveolar lavage fluid (BAL) and interstitial peribronchial and perivascular areas are required for the asthmatic phenotypes by engineering eosinophil-lacking PHIL mice in which expression of cytotoxic diphtheria toxin is congenitally driven by the eosinophil peroxidase gene promoter. G α i2-dependent CCR3 signaling promotes transendothelial migration of eosinophil [10].

When B16F10 melanoma cells were injected through the tail vein after aerosol challenges, numbers of metastatic foci on the pulmonary surface were increased by threefold [11]. T cell depletion by anti-CD4 antibody (GK1.5), corticosteroid inhalation and G α i2-KO condition, but not the PHIL background, abrogated the enhanced metastasis. Therefore, it is not eosinophils by themselves but CD4⁺ T-cell-dependent soil preparation to allow transendothelial migration of cells that appears to play a critical role for allergy-facilitated lung metastasis. The same group provided data of 176 breast cancer patients with lung metastasis. Approximately 13% suffered from asthma but with no corticosteroid treatment, which is about twice the frequency of asthma in a random population of American females.

15.3 Disruption of Local Homeostasis

CC10, alternatively called club cell secretory protein (CCSP) or uteroglobin (UG), is secreted by club cells in a steroid-inducible manner. CC10-KO mice are susceptible to LPS inhalation with increased leukocytes in BAL 24 h after LPS [12]. The concentration gradient of S100A8 was detected in the lungs down to blood in CC10-KO mice [13]. CC10-KO enhanced lung metastasis after injection of B16F10 melanoma cells through the tail vein. Although S100A8 was not expressed in B16F10 cells, they expressed RAGE whose ligand includes AGE and S100A8/S100A9. An anti-RAGE siRNA-mediated knockdown of RAGE in B16F10 prior to the tail vein injection suppressed lung metastasis. I assume that CC10-KO lungs are already cultivated by constant assaults of bacteria with LPS for the expression of S100A8 and S100A9 before tumor cell injection.

15.4 Disruption of Systemic Homeostasis

15.4.1 *VEGFR2-CN-NFATc Homeostatic Feedback by DSCR-1*

The calcineurin-NF-ATc pathway has been known as located downstream of antigen-presenting cell engagement to T cells but also is activated individually by the VEGF-VEGFR2 and thrombin-PAR-1 systems [14]. Both exert vascular permeability as I stated earlier. From cDNA microarray experiments of HUVEC stimulated by VEGF and thrombin, Down syndrome critical region-1 (DSCR-1, also called calcipressin 1, MCIP-1, and adapt 78) was found dramatically induced by 22.3-fold and 17.7-fold, respectively at 1 h [15]. It turned out that DSCR-1 inhibited nuclear localization of NF-ATc in HUVEC, i.e., inhibited calcineurin activity by direct interaction. The NF-ATc-mediated transcription induces expression of cytokines, such as IL-6, but simultaneous induction of the inhibitory molecule DSCR-1 forms a negative feedback loop to brake hyperactivation of the cytokine storm.

The expression of ICAM-1, E-selectin, and VCAM-1 were negatively affected by more than 50% in thrombin-stimulated HUVEC infected with DSCR-1 adenovirus [16]. Not only the mRNA expression level but also number of genes was down-regulated by DSCR-1. The same author stated that 52 of 172 thrombin-activated genes in HUVEC were down-regulated by DSCR-1 overexpression. Given that both VEGFR2 and PAR-1 provoke inflammation, the autoinhibitory mechanism serves to avoid an overshoot and maintain the oscillating physiological levels of calcineurin-NF-ATc activity. Therefore, this also can be called homeostatic inflammation.

Adenovirus-mediated gene transfer of DSCR-1 in B16 melanoma xenograft at approximately 50 mm³ tumor size, in which angiogenesis was actively taking place, inhibited the tumor growth. However, syngrafts of murine renal carcinoma (RENCA) and colon cancer (MC26) in DSCR-1⁻ KO mice displayed diminished tumor growth with significant reduction in SMA⁺CD31⁺ vessels [17]. Mechanistically, constitutive activation of calcineurin not only induced precocious NF-ATc nuclear localization in a cyclosporine-inhibitable manner but also dephosphorylated BAD to activate the apoptosis pathway in endothelial cells. Cerebral hemorrhage was detected in 15% of DSCR-1-KO at embryonic days 10 and 12 (E10 and E12).

Those experiments tell us important logics in homeostasis. There are several modes in negative feedback by molecules downstream, including catalytic inhibition of the molecule upstream by keeping the cells alive and cancellation of the biological outcome, such as growth, by eliminating the cells by apoptosis. It depends on the developmental stage of embryos and tumors. Another example of endothelial homeostasis is VEGF. Anti-VEGF antibody also resulted in side effects, such as brain hemorrhage [18]. VEGF-KO mice are embryonic lethal [19].

In ectopic syngraft model with eGFP-labeled B16F10 melanoma cells, premetastatic lungs were assumed by 12 days after subcutaneous implantation [20]. They found fibrinogen-fibrin deposit generated by vascular instability that is caused by expression of angiopoietin 2 (Ang2), MMP3, and MMP10. They could hardly detect up-regulation of VEGF in the lungs and claimed that both TGF β and TNF α were responsible for up-regulation of those molecules, siRNA-mediated knockdown of which resulted in suppressed infiltration of both myeloid cells and tumor cells. Ang2 is usually expressed in vascular remodeling sites in which vessels are more plastic with loosened endothelial junctions. Our experiments showed that blocking TGF β and TNF α individually suppressed up-regulation of S100A8 and S100A9 in premetastatic lungs of tumor-bearing mice.

When B16F10 melanoma cells were injected via the tail vein to Dscr1-lacZ mice, in which LacZ expression was engineered to manifest the calcineurin-NF-ATc activity, tyrosinase mRNAs were not detected until day 7 after tumor cell injection. Because it could be detected as early as days 3–5 before tumor cell arrival, they assumed the period as the premetastatic period. Interestingly, B16F10 injection in DSCR-1-KO mice significantly enhanced lung metastasis as judged by day 10. Orthotopic RENCA tumor model and LLC footpad model also aggravated lung metastasis in DSCR-1-KO mice, while growth of the primary tumor was suppressed as just described. The difference between the primary sites and metastatic lungs turned out to be Ang2 expression in the lungs without endothelial apoptosis. Up-regulation of both VEGF and activated VEGFR2 was recognized in the tumor-injected DSCR-1-KO mice.

15.4.2 *Inflammation in Vessels*

I have repeatedly stated the importance of vascular permeability in inflammation (Fig. 15.1; see also Chap. 14). What will happen when endothelial cells are systemically injured? It has been intensively studied from the standpoint of hypertension and vascular tone. Vascular tone is how much a vessel is constricted compared with maximum dilatation with largest lumen diameters. For example, angiotensin II (ATII) (amino acids 1–8) increases vascular tone, while by atrial natriuretic peptide (ANP) decreases it.

An early study in 1972 showed that ATII induced vascular permeability with interstitial edema in rabbit aorta and dermis in 30 min with Evans Blue as an indicator, which was accompanied by widening of inter-endothelial gaps by endothelial contraction [21]. ATII-stimulated vessels secrete PGE, which further increases permeability and induces vasodilatation.

ATII infusion for 14 days in mice displayed hypertension, reduced acetylcholine-mediated relaxation, increased maximum response to norepinephrine, hypertrophic remodeling with prominent macrophage infiltration, Nox2 up-regulation, and NF κ B activation [22]. Those are attenuated in Op/Op mice

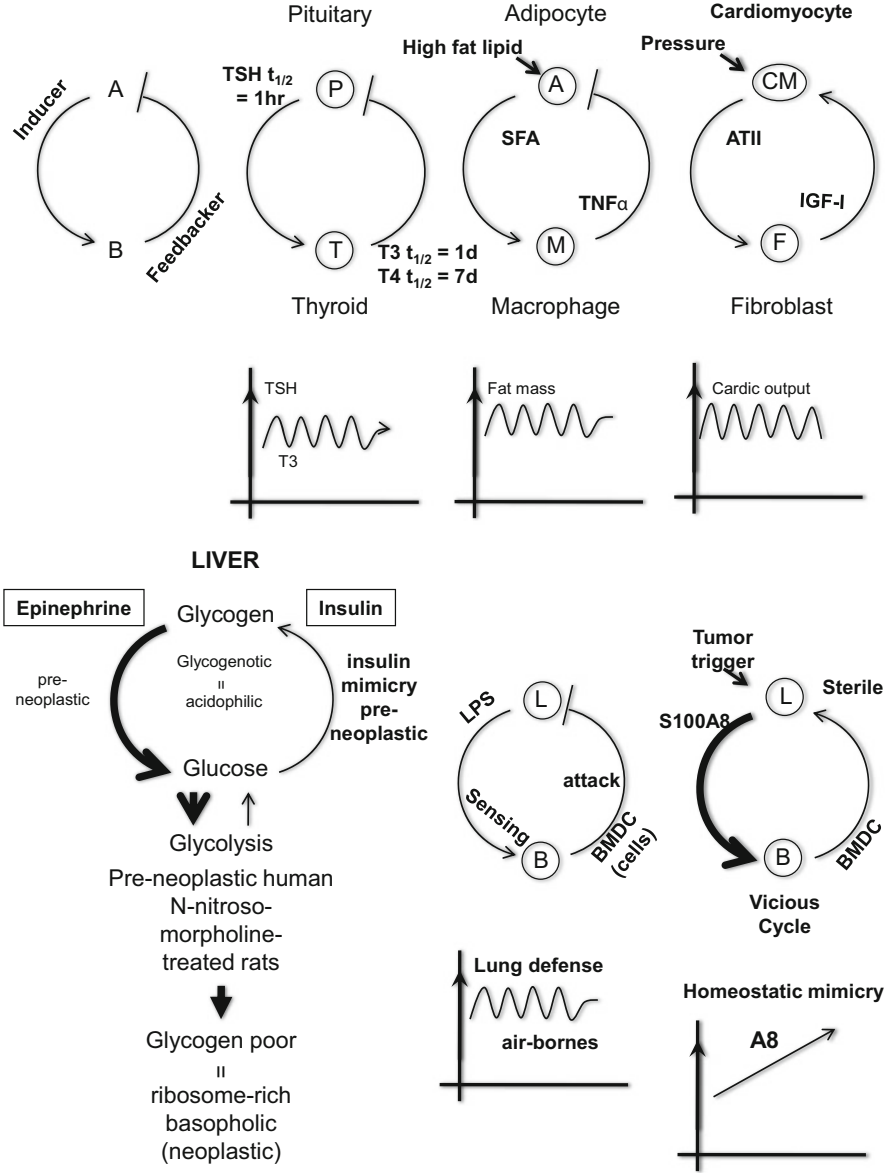


Fig. 15.1 Homeostatic mimicry

that are deficient in M-CSF showing monocytopenia and macrophage deficiency. In the same vessel injury model under the background of LDL-R-KO to induce hypercholesterolemia, development of atherosclerosis and aortic aneurysm was reduced by whole-body genetic deletion of ATII type 1a receptor (AT1a) [23]. The ATII-induced inflammation in vessels is accompanied by leukocyte

migration through activation of endothelial AT1a of both endothelial cells and vascular smooth muscle cells (VSMC) and the stimulated VSMC secretes the potent permeability factor VEGF and stimulated endothelial cells secrete chemokines, including CCL2, RANTES, MIP-1 α , and IP-10 [24]. Conversely, VEGF-induced vascular permeability is blocked by pharmacological inhibitors against ATII receptor. Here the vasoconstrictor induces inflammation-mediated increase of vascular tone.

In the renin-angiotensin system (RAS), angiotensinogen is processed to ATI (amino acids 1–10) by renin, which is further cleaved by ACE to ATII. Both ATI and ATII are inactivated by ACE2, a homolog of ACE, to AT (amino acids 1–9) and AT (amino acids 1–7), respectively. ACE2 negatively regulates RAS. Although the baseline levels of pulmonary ATII were not changed in ACE2-KO mice, lung injury by acid (see Chap. 6) significantly augmented its levels in ACE2-KO mice, which was accompanied by severe lung edema due to enhanced pulmonary vascular permeability as judged by Evans Blue but not to changes in pulmonary perfusion pressures. The high permeability was abrogated in AT1a-KO mice or pharmacological inhibitor for AT1 receptor [25, 26].

When B16F1 melanoma cells were intravenously injected, lung metastasis was reduced in AT1a-KO mice [27, 28]. Conversely, oral administration of ATII enhanced the metastasis. The authors showed an engagement between B16F1 cells with ATII-induced expression of P-selectin glycoprotein ligand-1 (PSGL-1) and platelets expressing P-selectin prompted VEGF release from the platelets. It is likely that ATII not only stimulates pulmonary endothelial cells to induce inflammation but also B16F1 cells to induce PSGL-1 expression to facilitate the engagement. The important point is that AT1a-expressing tumor cells may reach the premetastatic lungs and wait for an angiogenic switch, which could be mediated by ATII that plays a homeostatic role in the lung microenvironment. Macrophages also express ACE to generate ATII [29].

TNF α can induce vascular permeability as stated before. Transgenic mice of TNF α exhibited lung emphysema and severe pulmonary hypertension (PH) with vascular remodeling, such as interstitial thickening and perivascular fibrosis [30]. Because nitric oxide (NO) at 25 ppm failed to restore the right ventricular pressure, PH was not due to sustained vasoconstriction. In a skeletal muscle, TNF α infusion induced increased capillary permeability but only a minor effect on vascular tone [31]. The vascular permeability factor eventually increases vascular tone after sustained TNF α -induced inflammation.

Knockout mice of NPR-A, one of the receptors for ANP, displayed systemic hypertension [32]. However, LPS-induced vascular permeability in the lungs could be partially attenuated by ANP infusion even in NPR-A-KO mice, suggesting that ANP also may use other receptors, such as NPR-C, for pulmonary barrier protection [33]. Therefore, ANP not only decreases vascular permeability but also decreases vascular tone, i.e., causes vasodilatation. Lung metastasis induced by intravenous infusion of B16 melanoma cells after pretreatment with LPS was reduced by 75% by ANP infusion prior to the tumor cell challenge [34]. Pretreatment with ATII also

enhanced lung metastasis, which was inhibited by candesartan, an AII receptor blocker [35].

15.4.3 AA Metabolites

Now I state on arachidonic acid (AA) metabolites in vascular tone. In endothelial cells, AA is metabolized to (1) PGI₂ by cyclooxygenase (COX), (2) 12- or 15-HETE (hydroxyeicosatetraenoic acid) by lipoxygenase (LOX) and 11, 12- or 14, 15-EET (epoxyeicosatrienoic acid) by cytochrome P450 epoxigenases like CYP2C8 and CYP2J2. The EETs are subsequently metabolized into DHET (dihydroxyeicosatrienoic acid) with reduced biopotency by soluble epoxide hydrolase (sEH) (see Fig. 1.4 in Chap. 1).

Endothelial cells decrease their vascular tone individually by PGI₂, NO, and endothelium-derived hyperpolarizing factor (EDHF), such as EET [36]. Independence of those factors can be understood by non-NO and non-PGI₂-mediated relaxation even in the presence of pharmacological inhibitors against NOS and COX. To complicate the circumstances, many factors participate in their release. For example, histamine induces release of PGI₂ and EET. EET also is released by acetylcholine and bradykinin. EET opens Ca²⁺-activated K-channel and hyperpolarizes the membrane.

Syngeneic xenograft tumor models, including B16F10 melanoma, T241 fibrosarcoma, and LLC in the background of Tie2 promoter-driven CYP2C8, CYP2J2 transgenic (tg) and sEH-KO mice to achieve high levels of EET in endothelial cells exhibited dramatic increase in the tumor growth with enhanced tumor angiogenesis as judged by CD31-positive cells [37]. Transgenic mice of Tie2-sEH gave opposite effects. An enhanced corneal tumor angiogenesis was observed after systemic administration of 14,15-EET. In addition, stimulation of metastasis after removal of LLC primary tumor was further augmented in the background of those EET-high mice. I underline that they observed that the affected metastatic organs were multiple, including lymph nodes, liver, and lungs even without removal of the primary tumor. The EET-high tumor-bearing mice after tumor removal showed high levels of plasma VEGF and the resected tumor expressed high VEGF mRNAs. Most importantly and intriguingly, the authors performed parabiosis experiments to share circulation between, for example, a tumor-bearing Tie2-sEH-KO mouse as a donor with high EET and a Tie2-sEH-tg mouse as a recipient with low EET. This parabiosis gave enhanced tumor growth but the opposite condition, i.e., tumor in EET-low mice failed to enhance it. The shared high levels of EET were not sufficient to enhance tumor growth and endothelial EET levels were critical. It also was the case in metastasis. A tumor-bearing Tie2-CYP2C8-tg mouse with high EET failed to enhance metastasis in the recipient Tie2-sEH-tg mouse, demonstrating that endothelial EET levels control the metastatic microenvironment.

EET is produced in endothelial cells and therefore it appears to circulate and act on endothelial cells of the whole body. However, even in conditions where EET

could work in an endocrine manner, local actions in each endothelial cells appear to control its bioactivity. Both transgenic and KO engineering just provides artificial circumstances in which the endothelial EET simultaneously affects all endothelial cells of the whole body.

15.5 Obesity

Benign fat tumors are functionally malignant (Fig. 15.1; see also Chap. 14). A similarity can be recognized between cancer and adipose tissue. CCR2- and TLR4-promoted myeloid cells are mobilized in the primary tumor sites, premetastatic sites, and adipose tissues in obesity. The accumulated macrophages facilitate the tumor proliferation in the primary sites and hypertrophy of the adipocytes. Therefore, adipose tissues can be assumed to be benign tumors, because adipocytes do not make metastatic progression to different locations of adipose tissues nor circulating bone marrow-derived cells differentiate into adipocytes. Analyses of adipose tissue of mice that underwent BMT with GFP⁺ bone marrow revealed that there were no GFP⁺ cells that also were positive perilipin, an adipocyte marker [38].

15.6 Homeostatic Mimicry in Cancer

15.6.1 *Insulin Mimicry in Preneoplastic Foci in Liver*

A situation in which tumor cells utilize host homeostatic systems was proposed by Peter Bannasch in 1997 [39] (Fig. 15.1). The glycogen storage system in liver is homeostatically regulated by glycogenolysis by epinephrine and glucagon and glycogenesis by insulin. As I stated in Chaps. 10 and 12, glycogenolysis in liver and active glycolysis are metabolic hallmarks in cancer. However, an early preneoplastic event common to both chronic liver disease patients with a high risk for hepatocellular carcinoma and N-nitrosomorpholine-treated chemical hepatocarcinogenesis model in rats is glycogenotic activity that mimics the insulin effect [40]. This also can be recognized as a homeostatic response to a driving force for glycogenolytic activity that still is latent.

A similar phenomenon in angiogenesis is observed. Semaphorin3A (Sema3A) is one of the endogenous angiogenesis inhibitors by signaling through cellular cytoskeleton (see Chap. 16). In biopsy samples of human uterine cervical cancer patients, Sema3A was highly expressed in the epithelial cells and some endothelial cells in high-grade dysplasia called CIN-3, which was totally absent in cervical squamous cell carcinomas (SCC) [41]. This was supported by the HPV/E2 mouse model in which cervical tumor progression could be time-dependently observed from low-grade (CIN-1/2), CIN-3 lesions, and SCC. Sema3A was highly expressed

in endothelial cells in CIN-3 lesions. It is likely that the up-regulation of Sema3A is a homeostatic response against the angiogenic switch by the tumor before full tumor angiogenesis.

15.6.2 LPS Mimicry in Premetastatic Lungs

15.6.2.1 Roles of S100A8 (see Figs. 15.1–15.3)

The idea of LPS mimicry is based on the demonstration (see below) by us and other group that MD-2, the co-receptor of TLR4 (see Fig. 5.3 in Chap. 5), binds not only LPS but also S100A8 and SAA3.

In biochemical levels, we used full-length S100A8 and SAA3 proteins expressed in and purified from mammalian cells, and synthetic peptides of various lengths theoretically to avoid contamination of LPS. Surface plasmon resonance analyses revealed that K_d is 10 nM for mammalian S100A8 and 0.356 nM for mammalian SAA3 when tested with baculovirus-purified TLR4/MD-2 proteins [42]. The apparent K_d between TLR4/MD-2 and lipid A was 3 nM (see Chap. 5). The synthetic peptide of 2–89 amino acids of S100A8 binds MD-2 purified from baculovirus with K_d 0.73 μ M and stimulated cell migration in vitro in a manner dependent on TLR4, MD-2, and MyD88. The binding domain is localized in the C-terminal 30 amino acids S100A8 [60–89], which is supported by the results of docking simulation [43]. Overlapping peptide scanning of 15 amino acids of SAA3 revealed that amino acids 43–57 bind the MD-2 with K_d 30 μ M and stimulated cell migration dependently on TLR4, MD-2, and MyD88. Injection of the bioactive 15-mer to achieve a serum concentration around the K_d value in tumor-non-bearing mice could mobilize BMDC to the lungs in an MD-2-dependent manner [44].

When human peripheral mononuclear cells were stimulated by either 1 μ g/ml of *E. coli*-derived LPS or purified S100A8 with endotoxin <0.01 pg/ml for 24 h and the culture supernatants were subjected to cytokine array, both induced for example CCL2 but IP-10 and CXCL12 were induced only by LPS. Cytokines specifically induced by S100A8 were not found [45].

The up-regulation of S100A8 and S100A9 in premetastatic lungs is dependent on VEGF and TNF α , both of which are produced by the primary tumor [46]. The lung metastasis is blocked by anti-S100A8 antibody or TLR4-KO. Let us consider pneumonia. LPS in the alveolar space promotes permeability in epithelial barrier. Anti-S100A8 antibody can suppress transepithelial migration of leukocytes from the interstitial to alveolar space. Given the bacteriocidal roles of leukocytes in the alveolar space, it is reasonable for bacteria to promote epithelial permeability for invasion and for leukocytes to counter-migrate. Because anti-S100A8 antibody can block transendothelial migration of leukocytes from circulation to interstitial space, S100A8 is likely to contribute to extravasation of leukocytes in physiological and of tumor cells in pathological condition.

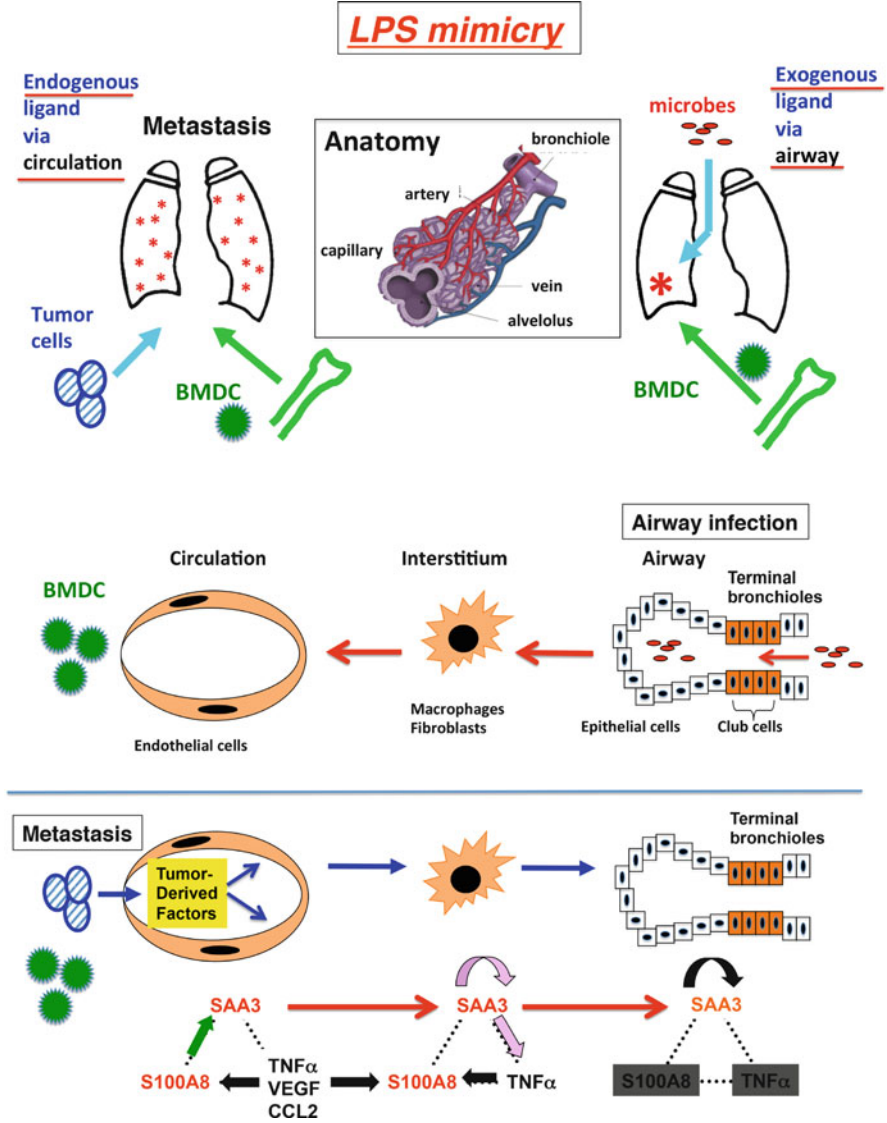


Fig. 15.2 LPS mimicry. Lungs have interphase between circulation and airway with paracrine signaling. Exogenously borne microbes through the airway induce mobilization of bone marrow-derived myeloid cells (*BMDC*) to the lungs. The triggering mechanism may involve microbial LPS that activates TLR4 in club cells in terminal bronchioles. The paracrine signaling goes in the direction to the circulation side. Expression of endogenous ligands, such as S100A8 and SAA3, in endothelial cells in sterile premetastatic lungs is induced by primary tumor-derived growth factors, such as CCL2, from the circulation side and the paracrine signaling goes in an opposite direction from the circulation to airway side to result in amplification of SAA3 in club cells. Bone marrow may misrecognize those endogenous ligands as LPS derived from lung infection and mobilize the myeloid cells there to battle against the phantom microbes

Leukocyte extravasation requires their initial attachment with endothelial cells. Bone marrow leukocytes are known to up-regulate Mac-1 (CD11b-CD18) expression in response to not only LPS but also other inflammatory factors including TNF α , LTB $_4$, and C5a. In neutrophils, in which roughly 40% of cytosolic protein is S100A8 and A9, native S100A8-S100A9 heterodimers purified from neutrophils failed to enhance adhesion to fibrinogen whose receptor is Mac-1 at concentrations up to 2 μ M, whereas *E. coli*-expressed S100A9 could do so by changing CD18 (= β 2 integrin) into an active form that can be recognized by monoclonal antibody mAb24 [47]. If the recombinant S100A9 protein was contaminated by LPS, it should be the LPS activity via TLR4. If not, the activity should have been mediated by a receptor that binds S100A9 but not S100A8; the most likely one is CD147 (see below) whose activation results in expression of TNF α that may work in a paracrine fashion.

S100A8 and S100A9 were claimed to be in vivo substrate of MMP9 whose knockout reduced lung metastasis [48].

The Peter Angel group nicely engineered hepatocyte-specific conditional double transgenic mice of S100A8 and S100A9, which displayed no disease phenotypes including inflammatory lesions [49]. Both proteins were detected weakly in the liver but not increased in the serum from the baseline. Interestingly, they showed roughly twofold up-regulation of serum concentration of CXCL1 with systemic enrichment of Gr1 $^+$ S100A8 $^+$ monocytic cells in circulation and passively in liver. Given that S100A8 was up-regulated in the serum of tumor-bearing mice in our experiments, S100A8 induction of CXCL1 is sufficient for leukocyte mobilization from the bone marrow. I am eager to see if portal injection of TLR4 $^-$ or RAGE-expressing cells can augment liver metastasis. For CXCL1, also refer to Chaps. 12 and 16.

Plasminogen activator inhibitor-1 (PAI-1) (see Chaps. 3 and 12), which is induced by LPS or ATF3 activation, plays a defensive role in pneumonia by facilitating transepithelial migration of leukocytes into the air space. Both S100A8 and SAA3 can potentially activate ATF3 (see below). Therefore, the defensive mechanism against bacteria by mobilizing leukocytes is <hijacked> by tumor cells. Just like leukocytes moving toward LPS, tumor cells move toward those endogenous TLR4 ligands in the lungs resulting in metastatic dissemination. PAI-1 inhibits plasminogen activator responsible for generating plasmin, which can activate MMP9 required for metastatic microenvironment. This seems paradoxical. However, S100A8 and SAA3 cause vascular permeability and fibrin-fibrinogen deposition, which as a next step activates fibrinolytic events.

15.6.2.2 S100A8 and the Eph-Ephrin System

S100A8 induces ephrin-A1 expression [50]. The original finding was that LPS induces TNF α , which then promotes expression of ephrin-A1 in HUVEC. The prototype of the Eph (The cDNA was originally isolated from a nude mouse transplantable tumor named erythropoietin-producing hepatoma) family of receptor

tyrosine kinases, now called EphA1, was discovered by me in 1987, and currently the family is grouped into EphA with ten members and EphB with six members. Their ligands are either GPI-anchored ephrin-A1 through A6 or transmembrane ephrin-B1 to B3 proteins, respectively. The system forms a counter-receptor system giving both forward (ephrin to Eph) and reverse (Eph to ephrin) signaling. The family members play important roles in many biological settings, including EphB4-ephrin-B2 in arteriovenous differentiation, ephrin-B2 in VEGFR2 activity, and EphA3/A5-ephrin-A2/A5 in neuronal pathfinding.

Systemic administration of LPS mimics bacterial assaults from the most exposed organs, i.e., lungs sensing air-borne LPS and liver responsible for perception of gut-derived LPS via portal vein. The misrecognition by those organs was reflected in the time-dependent up- and down-regulation of both EphA1/A2 and ephrin-A1 expressions, which is presumably involved in vascular permeability to allow leukocyte extravasation there [51]. A small elevation of LPS from whatever tissues attacked by bacteria is homeostatically controlled by this way.

We have shown that the EphA1 and EphA2 can bind the membrane-bound ephrin-A1 in lung endothelial cells serving as adhesion molecules in a manner independent of its tyrosine kinase activity [52]. However, a soluble form of ephrin-A1, which is released from the primary tumor by ADAM12-mediated shedding, reaches in an endocrine fashion and disrupts the pulmonary vascular barrier disorganizing VE-cadherin. We also have shown that the ephrin-A1-Fc recombinant soluble protein causes contraction of endothelial cells in a tyrosine kinase-dependent manner through the SAM domain binding to integrin-linked kinase and the subsequent RhoA-ROCK pathway [53]. In both EphA1-KO and EphA2-KO mice, vascular permeability in the lungs was actually increased. The information indicates the disruption of molecules that usually participate in lung homeostasis as adhesion machinery causes inflammation in the lungs, i.e., vascular permeability and cell migration. The critical mechanism is likely switching between on and off state of the EphA1 tyrosine kinase activity.

Therefore, the mechanisms of premetastasis cannot be explained without putting into consideration of molecules directly involved in lung homeostasis. The classical danger, such as invading bacteria or tumor cells and necrotic cells, are absent in the premetastatic lungs, but both vascular permeability and cell migration actively take place as in the case of physiological conditions. However, their levels are higher than those in physiological circumstances (see below). After arrival of tumor cells, which is the true danger, danger hypothesis-based events continue as found in the primary tumor.

15.6.2.3 A Single Match Can Start Fire

What is the mechanism by which to explain the sustained expression of S100A8 and SAA3 in the lungs while their serum levels decrease 10–14 days after implantation of tumor cells? Detailed analysis of stimulation and expression pattern of S100A8, SAA3, and TNF α revealed that the triggering mechanism is primary tumor-secreted

CCL2 that activates CCR2 in the hyperpermeable regions in the lungs to induce S100A8 expression in the endothelial cells. S100A8 from endothelial cells is secreted into the interstitium where macrophages are located and stimulated to secrete SAA3. The interstitial SAA3 then stimulates TLR4 expressed in club cells in the terminal bronchiole regions, a preferential site of lung metastasis. Club cells then express SAA3, which stimulates their own TLR4 resulting in autoamplification of SAA3 [54] (Fig. 15.2). At this stage, the paracrine cascade starting from CCL2 may be dispensable for the establishment of premetastatic milieu even if the primary tumor is removed. It is assumed that TLR4 inhibition may be one of the reasonable ways to put an end to the SAA3 autoamplification.

An initial LPS exposure is known to induce tolerance against subsequent LPS challenge. The mechanisms involve TLR4-induced activation of aryl hydrocarbon receptor (AhR) whose ligands include dioxin of environmental origin and endogenous kynurenine, a product of tryptophan catabolism by indoleamine 2,3-dioxygenase 1 (IDO1) that also is induced by TLR4 activation, as manifested by decreased serum levels of TNF α and IL-6 [55]. AhR-KO mice were more sensitive to endotoxemia by impaired mitigation of the TLR4-NF κ B signaling by AhR. AhR is expressed in club cells that metabolize xenobiotics and can autoamplify SAA3. By utilizing AhR-expressing primary hepatocytes derived from AhR-KO mice, it was shown that a potent AhR agonist TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) repressed SAA3 expression induced by IL-1 β and IL-6 [56]. The authors showed that similar effects were observed with different AhR agonists, such as benzo[a]pyrene and naphthoflavone, and that the mechanisms involved inhibited recruitment of p65RelA and C/EBP β to the SAA3 promoter. However, ongoing studies in our laboratory have shown that neither S100A8 nor SAA3 lack this feedback (Tsukahara and Maru, unpublished results).

Readers should remember that CCL2 is also involved in homeostatic inflammation in alveolar recruitment of monocytes (see Chap. 14). We have shown that both S100A8 and CCL2 can induce vascular permeability in the lungs. I will review interesting experiments by Jeffrey Pollard group [57]. They defined inflammatory Ly6C⁺ (therefore Gr1⁺) (see Chap. 12.6.3.2) and resident Ly6C⁻ (Gr1⁻) monocytes sharing CD45⁺CD11b⁺CD115⁺ phenotypes (CD115 is CSF1R and almost all CD115⁺ cells are Ly6G⁻), which were sorted from CSF1-R-GFP transgenic mice to track down after adoptive transfer into MMTV-PyMT mice. While Gr1⁻ monocytes were recruited in the primary tumor, Gr1⁺ monocytes were mobilized to the late-stage lung metastatic lesions and forced pulmonary metastatic nodules by intravenous injection of a PyMT-induced mouse mammary tumor cell line (Met-1), and intriguingly lungs 7 h after intravenous injection of Met-1 cells before extravasation in the lungs, but not 7-week-old PyMT mice with yet premalignant mammary tumors presumably without expression of CCL2 even if they make an entry into circulation. High levels of CCR2 expression were observed in the recruited Gr1⁻ monocytes. Notably, CCL2 expression levels in tumor cells were homogenous in the metastatic lung nodules but heterogenous in the primary tumor. Therefore, CCL2 expression appears to confer a metastatic trait on the tumor cells. Given that even CTC expressing CCL2 alone without primary tumor induced Gr1⁺

monocytes in the lung, augmented levels of CCL2 in circulation may be misrecognized by Gr1⁺ monocytes in the bone marrow as a danger producing CCL2 in the lungs. The recruited monocytes are destined to prepare premetastatic soil by producing VEGF in contact with CCL2-secreting tumor cells and VEGF works in concert with CCL2 to facilitate tumor cell extravasation into the lungs. They showed that myeloid cell-specific conditional knockout of VEGF by the tamoxifen-inducible system linked to the CSF1-R promoter abrogated the tumor cell seeding in the lungs. VEGF by itself was not required for the recruitment of Gr1⁺ monocytes since VEGF-null monocytes were recruited at similar levels to the control.

15.7 TLR4-Deficient Phenotypes

TLR4 is a double-edged sword but seemingly defensive.

The TLR4 polymorphism D299G in the extracellular domain showed reduced LPS response with a greater risk of sepsis and decreased ability for binding HMGB1, an endogenous TLR4 ligand (see Part II). The frequency of metastasis by 5 years after breast cancer surgery was 40% in D299G patients compared with 26.5% in those without polymorphism, suggesting the polymorphism is loss of defensive function [58]. Autosomal recessive mutations in MyD88 within the IRAK4-interacting domain, such as L93P result in failed interaction with IRAK4 leading to severe infection with pyrogenic bacteria during the early infancy [59]. Compared with reactions in the initial exposures to exogenous bacteria in neonates, those in early adolescence get less and less severe. Conversely, a gain of function mutation L265P in MyD88, which results in constitutive MyD88 signaling with production of cytokines, such as IL-6, can facilitate diffuse large B-cell lymphoma progression [60]. Thus, the TLR4-MyD88 pathway appears to be defensive against bacteria and tumor progression, but too much defensive reaction favors tumor progression.

LPS reduces the expression of CCL21 through the TLR4-SOCS3 pathway in high endothelial venules in lymph nodes, which results in less mobilization of CCR7-expressing lymphocytes as attackers to the lymph nodes [61]. In this case, TLR4 weakens the defense via CCL21. When tumor cells express CCR7, CCL21 promotes their mobilization through afferent lymphatic ducts that are dilated by VEGF-C. Here, CCL21 promotes the attack. In both cases, it is favorable for attack by tumor cells.

This apparent discrepancy can be easily understood by thinking of homeostatic mimicry and the hijacking idea. LPS mobilizes defensive BMDC. S100A8 also mobilizes defensive BMDC [62]. Mobilized BMDC participates in more S100A8 production but find no enemy or attackers to battle against in the premetastatic lungs. Overexpressed S100A8 mobilizes aggressive tumor cells expressing TLR4 in postmetastatic lungs. An siRNA-mediated knockdown of TLR4 in tumor cells

abrogated their lung metastasis [54]. At this stage, defenders are substituted with attackers.

TLR4 activation inhibited growth of breast cancer cells with wild-type p53 by increasing growth-suppressing IFN γ secretion but promotes growth of those with p53 mutations by expression proinflammatory cytokines, including IL-6 and CXCL1 [63].

As I just stated, both plasma S100A8 and SAA3 elevate in tumor-bearing mice 10 and 8 days, respectively, after subcutaneous implantation of tumor cells with recruitment CD11b⁺ myeloid cells in the premetastatic lungs. There are some differences between intravenously injected LPS and the endogenous TLR4 ligands produced in tumor burden. Reciprocal BMTs between wild-type and TLR4⁻KO mice can theoretically generate (1) BM TLR4⁻KO with most of the pulmonary population of cells, such as epithelial and endothelial cells are of wild-type, and (2) lung TLR4⁻KO with recruitment of a small population of wild-type cells from the bone marrow. When systemic LPS was administered at 0.5 mg/kg, neutrophil recruitment in the lungs was observed within 4 h as monitored by myeloperoxidase activity and chloroacetate esterase staining in the lungs, which was abrogated in TLR4⁻KO and lung TLR4⁻KO, but not BM TLR4⁻KO, E- and P-selectin double KO and CD18-KO [64]. LPS-activated TLR4 was reflected in the P-selectin expression in the lungs in totally wild-type and BM TLR4⁻KO mice but not TLR4⁻KO and lung TLR4⁻KO mice. Collectively, LPS stimulates TLR4 expression in the lung resident cells to induce neutrophil recruitment.

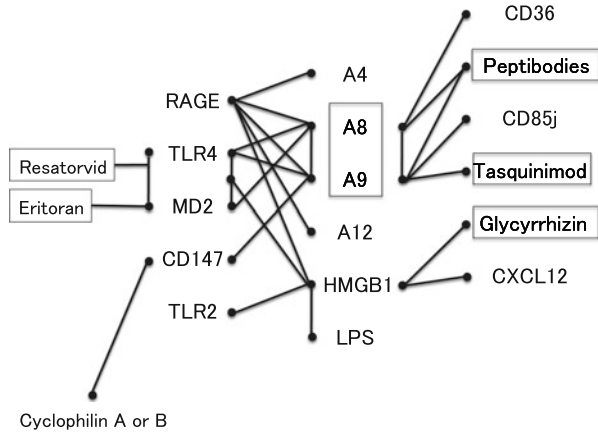
When similar experiments were performed by tumor challenge, which increases plasma levels of both S100A8 and SAA3, pulmonary recruitment of Mac1⁺ myeloid cells was impaired in TLR4⁻KO and BM TLR4⁻KO mice but not lung TLR4⁻KO mice, indicating that blood cell TLR4 is important, but the experiments failed to reduce the significance of lung TLR4. Because club cells, which express TLR4, are derived from BM, a mixture of TLR4⁺ and TLR4⁻ club cells was present in lung TLR4⁻KO mice.

15.8 S100 Family

More than 20 members are known in the S100 family proteins [65]. They are homologous to each other by 25–65% in amino acid levels. Information of the members with determined crystal structure revealed that they have an EF-hand motif and form antiparallel homo- or hetero-dimers. They can further associate to form high-order multimers. High-resolution NMR spectroscopy revealed that binding of Ca²⁺ opens up the hydrophobic pocket allowing their interaction with the C-type immunoglobulin domain of RAGE, i.e., Ca²⁺ molecularly switches the binding. This Ca²⁺ sensor is engaged in a variety of biological events by functioning in intracellular and/or extracellular space.

S100A8 can preferably form a hetero-dimer with S100A9. In peripheral blood cells from S100A9-KO mice, S100A8 proteins were under detection, although S100A8 mRNAs were present, suggesting that S100A9 proteins are responsible

Fig. 15.3 Multiligand/multireceptor system



for stabilization of S100A8 proteins in mice [66]. An opposite situation was reported in humans. Human S100A9 proteins were unstable in the absence of S100A8 proteins [67]. Both S100A8 and S100A9 proteins were initially identified in the synovial fluid of rheumatoid arthritis patients [68].

Corpora amylacea in prostate glands of prostate cancer patients mainly consists of amyloid (see Chap. 6) of S100A8 and S100A9 [69]. S100A8/S100A9 proteins purified from granulocytes showed a propensity to aggregate in vitro forming fibrillar structures in 8 weeks in a manner dependent on Zn^{2+} and Ca^{2+} as judged by atomic force microscopy and transmission electron microscopy [70]. Liquid chromatography-electrospray ionization mass spectrometry of corpora amylacea revealed the presence of hemoglobin subunits and myeloperoxidase of host origin as well as bacterial DNA sequences, strongly indicating the involvement of bacterial infection-induced inflammation in the formation of this crystal.

S100A8 expression is up-regulated not only in pre-metastatic lungs but also in synovial fluid in rheumatoid arthritis patients, serum from SLE and intestinal epithelial cells of Crohn's inflammatory bowel disease and most importantly in tumors [71, 72].

In contrast to most secretory proteins exported by the ER-Golgi pathway, unconventional, i.e., still unknown mechanisms underline the release of IL-1 β , IL-18, IL-33, proIL-1 α , FGF, and S100A8, all of which lack the leader sequence [73]. It is known that IL-1 β secretion depends on NALP3 inflammasome and caspase-1 activation [74].

In addition to soluble factors, such as TNF α and LPS, HIF-1 induces expression of both S100A8 and S100A9 by binding to their hypoxia response element (HRE) in the promoters in benign prostate epithelial hyperplasia BPH-1 cells and prostate cancer PC-1 and DU-145 cells [75]. Analysis of 145 patients who were subjected to radical prostatectomy revealed similar expression patterns of S100A8 and HIF-1 α in immunostaining and a negative correlation between the expression levels of S100A9 and the time required for recurrence.

Although dexamethasone alone failed to induce S100A8 expression in 20 h but enhanced LPS-stimulated expression in thioglycolate-elicited murine macrophages, it directly induced S100A8 expression in human blood monocytes. Synovial knee joint biopsy of RA patients revealed that S100A8-positive cell numbers increased 24 h after intravenous administration of methylprednisolone [76]. Up-regulation after steroid may imply that S100A8 is at least seemingly anti-inflammatory. However, the story is not so simple. For example, increased expression of S100A8 in acute lymphoblastic leukemia with MLL gene translocation confers glucocorticoid resistance on the patients. In this case, abundant intracellular S100A8 is assumed to interfere with glucocorticoid-elicited Ca^{2+} fluxes from the ER to mitochondria [77].

Two receptors are known for S100A8: TLR4 and RAGE [78, 79] (Fig. 15.3). More precisely, we showed that S100A8 binds the MD-2 coreceptor of the TLR4-MD-2 complex. Only N-glycosylated RAGE, which constitutes only 5% of total RAGE, can bind S100A8 [80]. From the RAGE side, it can bind the authentic ligand AGE, S100A6, and S100A12 [81, 82]. One of the unique features of S100 proteins is that they lack signal peptides usually essential for secretory proteins, but they are actually secreted actively and passively into the extracellular space by an as yet unknown mechanism.

Members that participate in tumor biology include S100A2, S100A4, S100A6, S100A7, S100A8, S100A9, and S100B [83]. Clinical correlation between the expression and invasive levels has been reported in S100A4 (also called metastasin) in particular. This is supported by two logical experiments, i.e., metastasis in MMTV-neu mice was enhanced when crossed with S100A4 transgenic mice, while that in MMTV-PyMT with S100A4-KO was suppressed, indicating that S100A4 is necessary and sufficient for the tumor metastasis although the mechanism is still unclear [84]. Both stromal cells and tumor cells are influenced in terms of expression levels of S100A4 in those tumor models.

Similar to S100A8, it is abundant in the synovial fluid of rheumatoid arthritis patients and in the psoriatic skin. Intraperitoneal administration of anti-S100A4 antibody in the human psoriasis xenograft in SCID mice was effective by reducing the epidermal thickness and Ki-67+ proliferating cells [85]. While expression of S100A4 in tumor cells induces their metastatic potentials, various types of cells, including T cells, DC, macrophages, fibroblasts, and myofibroblasts, are S100A4-positive in psoriatic skin. Secreted forms of S100A4 are thought to bind RAGE.

15.9 Issue of Multiligand/Multireceptor System

S100A8 and S100A9 can form homo-dimers and hetero-dimers, but their partial synthetic peptides do not necessarily do so (Fig. 15.3). I mean either monomer or homo-dimer by S100A8 and heterodimer by S100A8/S100A9. In 1999, Marion

A. Hofmann initially showed evidence of a linkage of S100 family with inflammation by S100A12 binding to RAGE whose authentic ligand is AGE (see Chap. 6) pathologically essential in diabetes mellitus [82]. Mice lack the S100A12 gene of which S100A8 may function in place.

In a skin carcinogenesis model induced by chemicals DMBA/TPA, S100A8, S100A9, MIP-1 α , MIP-1 β , and MIP-2 were up-regulated [86]. Those are target genes of NF κ B that is activated by RAGE or TNF α . Knockout mice of either TNF α or RAGE were resistant to this chemical carcinogenesis, suggesting sustained NF κ B activation by a positive feed-forward loop of S100A8/S100A9 to RAGE to NF κ B to S100A8/S100A9. Reciprocal BMT experiments with wild-type and RAGE-KO mice revealed that RAGE in bone marrow cells is responsible for carcinogenesis and dermal infiltration of neutrophils and macrophages. Now RAGE has at least five ligands, i.e., AGE, S100A6, S100A12 (only human), S100A8/S100A9, and HMGB1. Conversely, S100A9 has at least three receptors: RAGE, TLR4, and EMMPRIN.

Tomas Leanderson group has shown that human S100A9 and dimeric S100A8-S100A9 bound human RAGE with KD of 38 nM and 9.4 nM, respectively [87]. It is of note that S100A12 bound RAGE with KD at 90 nM. They also bound the human TLR4-MD-2 complex with KD of 2.1 nM and 3.8 nM, respectively. Because the binding was not inhibited by simultaneous application of MD-2 proteins, it is likely that S100A9 binds the TLR4 side.

Affinity isolation as spectrometry in search of binding proteins for S100A9 identified EMMPRIN also called CD147 [88]. S100A9 stimulation induced expression of CXCL1, TNF α , ephrin-A1, and MMP1 in the EMMPRIN signaling presumably via TRAF2. RAGE, another receptor for S100A9, failed to form a heterodimer with EMMPRIN. Intravenously injected EMMPRIN-expressing melanoma cells metastasized to the skin of S100A9 but not S100A8 transgenic mice with the epidermis-specific involucrin promoter.

Furthermore, pull-down experiments of human monocyte-derived dendritic cells after HIV-1 infection with Fc-fused CD85j, which belongs to the human leukocyte immunoglobulin-like receptor family, revealed binding of S100A9 [89]. They reported that stimulation of purified NK cells by recombinant tetrameric but not monomeric S100A9 proteins expressed and purified from *E. coli* in endotoxin-free conditions as they claimed increased production of TNF α and enhanced their anti-HIV-1 activity.

15.10 Endogenous Modulators

If homeostatic, LPS should have a feedback loop. See Fig. 5.3 in Chap. 5 for the fundamental TLR4 signaling cascade.

15.10.1 A20

Negative regulation by A20 was stated in Chap. 8.

15.10.2 ATF3

In addition, upon TLR4 activation, NF κ B-mediated ATF3 transcription takes place [90]. ATF3 directly associates with HDAC1 to inhibit TLR4-driven gene expression [90]. Although LPS stimulation induced expression of neutrophil chemoattractant CXCL1 in ATF3-KO mice, neutrophil recruitment in the ATF3-KO lungs was not observed [91]. Gene expression profile analysis revealed that TIAM2 was down-regulated in ATF3-KO, which was responsible for the migration defect.

In the OVA-induced model of bronchial asthma, ATF3 is up-regulated in the lungs. ATF3-KO increased the hyperresponsiveness, pulmonary eosinophilia, and Th2 cytokine production [92]. When you remember this model combined with lung metastasis by B16F10 melanoma cells (see above), you would expect aggravation of metastasis in the lungs. However, myeloid specific ATF3-KO or general ATF3-KO both reduced lung metastasis in the background of syngeneic orthotopic MMTV-PyMT mammary cancer model [93]. Interestingly, there was no difference in the primary tumor growth. Transcription factor ATF3 is activated in a variety of cells essentially by stress, such as hypoxia, IL-6, and TNF α . ATF3-activated macrophages, for example, switch the balance to M2 and directly activate MMP9 expression. This may be one reason to explain the metastasis-suppressing activity in the ATF3-KO mice.

15.10.3 CD11b

Activated TLR4 also induces PI3K- and RapL-mediated activation of CD11b, i.e., changing its conformation from an inactive to active state. As you remember that CD11b is an integrin α M β 2, this is an inside-out signal. This elicits a sequential activation of Src and Syk associated with ITAM [94]. Then Syk phosphorylates MyD88 and TRIF, to which an E3 ligase Cbl-b binds and ubiquitinates for proteasomal degradation. This eventually weakens the LPS-induced signaling by CD11b-mediated negative feedback.

15.10.4 Calcineurin

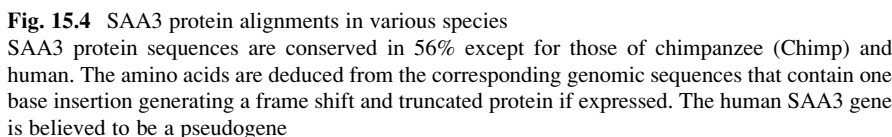
Calcineurin inhibitor, such as FK506, or an siRNA-mediated knockdown of calcineurin in RAW cells augmented the TLR4-NF κ B cascade-mediated expression of TNF α [95]. Conversely, a constitutively active calcineurin by deleting the autoinhibitory and calmodulin-binding regions reduced LPS-stimulated activation of NF κ B. Given that TNF α production by FK506 was reduced in macrophages deficient in MyD88, TRIF, TLR4, and TLR2 and that calcineurin co-immunoprecipitates with them, the mechanisms may involve interaction, direct or indirect, between calcineurin and those molecules.

15.10.5 Akt

LPS activates Akt. While TRAF6 located downstream of TLR4 signaling can serve as a direct E3 ubiquitin ligase for Akt important for its membrane translocation and activation in growth factor-activated tumor cells, Akt controls regulatory microRNAs [96]. For example, Akt up-regulates miRNA let-7e that is capable of repressing TLR4 expression within a few hours, which is restored to the baseline in 48 h. Akt1-KO macrophages displayed exaggerated responses to LPS.

15.11 SAA3 Exists in Humans

The deduced amino acid sequence portion between mouse and human SAA3 are highly homologous to each other, and a single nucleotide insertion in the human SAA3 exon2 results in a frameshift to generate a shorter SAA3 with a unique C-terminal SAA3 sequence of 12 amino acids. Interestingly, this type of evolutionary genetic alteration occurs in primates, including chimpanzees (Fig. 15.4). Because no expression of human SAA3 has been reported so far, it has been assumed to a pseudogene. Although we could not find human SAA3 transcripts in the database of the expressed pseudogenes in tumor cells [97], elaborate qPCP analysis of human lung cancer samples in our laboratory revealed that there exists a chimeric SAA2-SAA3 fusion mRNAs [98]. The human SAA2 exon3 is connected to the human SAA3 exon2 with roughly 200 kb between the 2 exons to yield the SAA2-SAA3 fusion protein that could be recognized by a monoclonal antibody against the unique sequence of human SAA3. The corresponding gene product activates Erk by binding and stimulating LOX-1, an endothelial cell-specific scavenger for oxidized LDL. The binding affinity was 206 nM as judged by ELISA between C-terminal synthetic 26-mer human SAA3 peptide and soluble LOX-1. Given that neither the SAA2-SAA3 purified from mammalian cells nor synthetic



15.12 Neonatal Switch from Bacteria to Endogenous Mediators

We have observed up-regulation of individually CCL2⁺ and CCR2⁺ cells in the lungs of LPS-treated but not PBS-treated germ-free neonatal mice. However, neonatal mice housed in the standard SPF conditions, in which they were exposed to LPS-containing bacteria, increased numbers of CCL2⁺ and CCR2⁺ cells were recognized even PBS-treated mice. In the SPF neonates LPS administration followed by LLC injection induced the tumor cell recruitment in the lungs, which was totally abrogated in CCR2-KO mice (Hiratsuka and Maru, unpublished data). Thus, neonatal exposure to exogenous LPS activates the CCL2-CCR2 system, which leads to the endogenous TLR4 cascade of the S100A8-SAA3 paracrine system.

A similar situation is known in SAA3 expression in adipose tissue through gut [99, 100]. Levels of SAA3 mRNA in epididymal adipose tissue and colon of germ-free Swiss-Webster mice were approximately ten- and sevenfold, respectively, higher than those raised conventionally. MyD88-KO reduced the SAA3 mRNA levels by sixfold compared with the wild type. LPS simulates TLR4 in the colon epithelial cells as well as macrophages to induce SAA3 expression. Serum levels of SAA3 in conventionally raised mice were higher than those in germ-free mice and LPS administration induced SAA3 expression in adipose tissue. Given that both authentic ligand LPS and endogenous one SAA3 bind TLR4 in adipose tissue macrophages to secrete SAA3 by a feed-forward mechanism. Thus LPS-triggered and autoamplified SAA3 production stimulates myeloid cell mobilization from bone marrow resulting in accumulation of macrophages in both tissues. The inflammatory response in both colon and adipose tissue is stabilized at homeostatic levels. We should pay attention to the mechanism by which LPS-induced inflammation in colon can spread to other tissue(s) if LPS can induce expression of endogenous TLR4 ligands, because they can function as LPS in bacteria-free tissues.

15.13 MDSC and Premetastasis

What are the roles in premetastatic lungs of MDSC that I stated in the Chap. 12? In our initial report of premetastatic lung establishment by S100A8, we monitored Mac-1⁺ cells. This population of cells includes CD11b⁺ Gr1⁺ cells, i.e., MDSC. In breast cancer ectopic xenograft model with 4T1 cells labeled by GFP, which causes spontaneous metastasis to the lungs, flow cytometric analysis and qPCR analysis of GFP of the lungs showed that mice did not have tumor cells in the lungs until 14 days after tumor implantation. Coculture of sorted MDSC with normal lung cells revealed that the number of IFN γ -producing macrophages was significantly decreased [101]. Histological analysis of the premetastatic lungs showed that costaining of CD11b⁺Gr1⁺ and MMP9⁺ cells. In addition, HUVEC cocultured with MDSC producing MMP9 disrupted VE-cadherin of HUVEC.

It is likely that MDSC cultivates the soil by reducing antitumor effect and by promoting pulmonary vascular permeability.

MDSC accumulate not only in the premetastatic lungs but also in other parts of the body, such as the spleen, by more than fivefold in 3 weeks after tumor cell transplantation. Analysis of the splenic population of MDSC revealed several interesting features: (1) MDSC failed to accumulate in tumors transplanted in S100A9-KO mice but instead CD8⁺ and CD4⁺ T cells infiltrated the tumor and 9 of 12 mice rejected tumor; (2) S100A9 overexpression induced MDSC accumulation by blocking myeloid differentiation via the Nox2 complex-mediated production of ROS.

Is S100A8-SAA3 expression necessary and/or sufficient for premetastatic soil establishment? Both anti-S100A8 and anti-SAA3 antibodies individually blocked lung metastasis as we reported previously [42, 46]. SAA3-KO mice showed no prominent phenotypes in metastasis experiments in our hands (Tomita T and Maru Y, unpublished results). Because S100A8-KO mice were embryonic lethal, we are currently trying to establish conditional S100A8-KO mice to uncover the homeostatic role of S100A8.

We have discussed on inflammatory conditions, including LPS administration and acute pneumonia, which promote lung metastasis. Knockout mice of uteroglobin, which is an anti-inflammatory protein also called CCSP or CC10 (see Chaps. 14 and 15), a specific marker of lung club cells, displayed up-regulated expression of lung S100A8 and enhanced lung metastasis when injected with B16F10 melanoma cells through the tail vein in a tumor nonbearing condition [13].

In mice with keratin 5-directed expression of $\text{IkB}\alpha$ to the epidermis (K5- $\text{IkB}\alpha$), inflammatory hyperplasia with S100A8⁺ neutrophil that dominated over F4/80⁺ macrophages took place followed by the development of well-differentiated squamous cell carcinomas at 4 months of age. Up-regulation of $\text{TNF}\alpha$, S100A8, and SAA3 was found in the skin lesions, which were sensitive to UVB at 500 J/m² to induce apoptosis [102]. The dermatitis and skin tumor formation were abrogated when K5- $\text{IkB}\alpha$ mice were crossed with TNFR1-KO but not CCL2-KO mice [103, 104]. Murine mammary cancer Met1 cells were subcutaneously implanted in K5- $\text{IkB}\alpha$ mice, and the tumor was resected to promote lung metastasis. In premetastatic lungs, CD11b⁺Gr1⁺ cell numbers were elevated but reduced after the resection. However, the resection induced development of macroscopic metastatic nodules in the lungs but not in the skin where premetastatic factors that we proposed, such as S100A8 and $\text{TNF}\alpha$, were constantly up-regulated (Tomita, Toftgard and Maru, unpublished data). Lung metastasis after resection can be explained by elimination of negative angiogenic switch on the tumor cells that reached the lungs before resection. However, the tumor cells could hardly reach the inflammatory skin, suggesting the presence of organotropic factors that control the lung metastasis.

15.14 Hijacking the RANKL-RANK System

Binding of receptor activator of nuclear factor κB (RANK) in osteoclast precursors and RANK ligand (RANKL) in osteoblasts induces osteoclast differentiation in the presence of M-CSF [105] (Fig. 15.5). The osteoclast precursors are derived from bone marrow hematopoietic stem cells and c-Kit⁺ M-CSFR⁺ CD11b low. This homeostasis in bone is hijacked by tumor cells to achieve bone metastasis by ectopically expressing both RANKL and RANK [106].

RANK stimulation elicits signaling through Akt and ERK, inducing cell migration but not cell proliferation or cell death. RANK is frequently expressed in breast

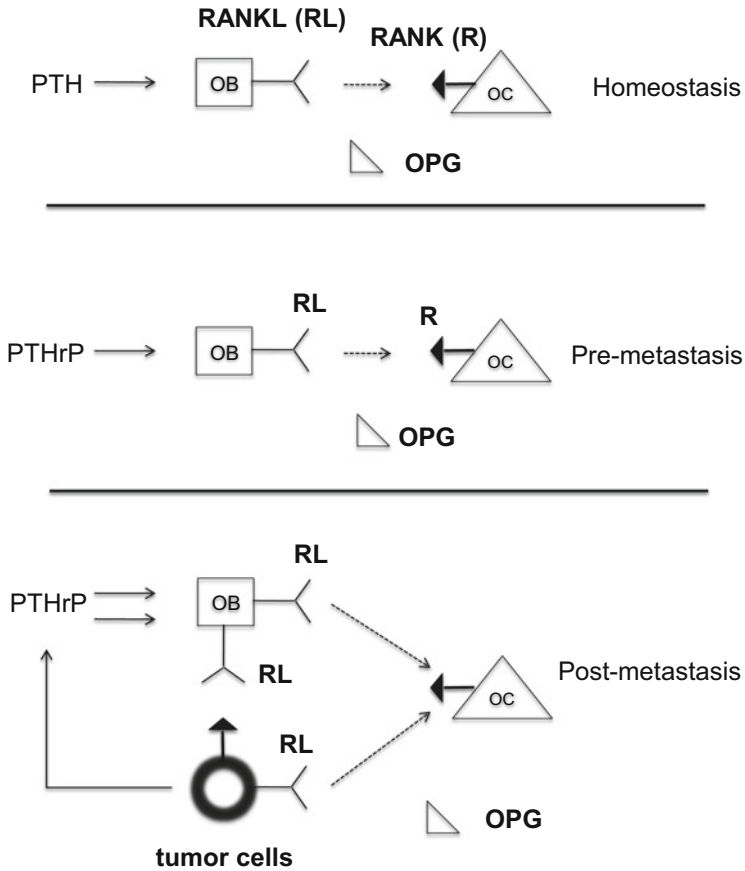


Fig. 15.5 RANK-RANKL in bone metastasis

and renal cell cancer [107]. RANK is also expressed in normal mammary gland epithelial cells. A report of 73 clear cell carcinoma of kidney showed that RANK, RANKL, and the soluble form of decoy receptor for RANKL called osteoprotegerin (OPG) were expressed in 82%, 66%, and 71%, respectively, as judged by immunostaining. Although roughly 30% of renal cell cancer patients develop bone metastasis, OPG-high patients were free of bone metastasis. Patients with strong expression of both RANK and RANKL had extramedullary metastasis to skin and liver.

RANK-expressing tumor cells migrate towards RANKL-expressing osteoblasts in bone. On arrival, RANKL in tumor cells and induced RANKL in osteoblasts by tumor cell-derived PTHrP stimulate RANK to induce active osteoclastogenesis and subsequent osteolytic bone metastasis. Given that RANKL is induced by LPS in

osteoblasts, endogenous TLR4 ligands, such as S100A8, derived from either myeloid cells or tumor cells might play a role in osteoclastogenesis [108].

Intracardiac injection of B16F10 melanoma cells that express high levels of RANK resulted in metastasis in bones, ovaries, adrenal glands, and brain. OPG not only inhibited RANKL-dependent cell migration of tumor cells *in vitro* but also suppressed bone metastasis *in vivo*. Intriguingly, however, metastasis to other organs was not affected. This information indicates that hijacking the RANK-RANKL system participates not only in the mobilization of tumor cells but also in organ specificity in metastasis.

In addition to tumor cells, LPS-activated TLR4-expressing neutrophils express RANKL. This also is the case in neutrophils from the synovial fluid of exacerbated rheumatoid arthritis patients in which endogenous TLR4 ligands S100A8 and S100A9 are abundant [109]. Therefore, RANKL not only in tumor cells but also in neutrophils participates in osteoclastic bone resorption.

COX-2 expression (see Chap. 1) is observed not only in a variety of tumor cells but also stromal cells. LPS induces PGE2 production in macrophages. In postmetastatic bone experiments with melanoma cells, we have shown by using coculture systems and gene knockout mice that B16 melanoma cell-osteoblasts contacts resulted in osteoblastic expression of RANKL in a membrane-bound (m) PGES-1 (see Chap. 1)-dependent manner, leading to osteoclast formation [110]. Bone metastasis was abrogated in genetic background of mPGES-1-KO or in the presence of an EP4 antagonist AE3-208 when melanoma cells were injected intravenously through the tail vein. Thus, in this case the RANK-RANKL system is hijacked by melanoma cell-stimulated activation of mPGES-PGE2-EP4 signaling in osteoblasts.

15.15 Connexin Hijacking

Another way to hijack homeostasis is found in one of the mechanisms of brain metastasis by breast cancer and melanoma. Connexin43 mediates homocellular communications in endothelial cells (see Chap. 3). Endothelial cell-specific knock-out of connexin43 resulted in dramatic NO elevation and hypotension suggesting that vascular gap junctions contributes to vascular homeostasis [111]. Intraperitoneal injection of LPS into mice also induced bladder connexin43 expression in bladder smooth muscle cells, which was largely blocked by an iNOS inhibitor. This suggests complicated mechanisms of connexin43 expression in a variety of cells in which NO generation systems are involved [112].

An EMT regulator Twist up-regulates connexin43 expression in breast cancer cells, which mediates heterocellular communication between the cancer cells and brain endothelial cells [113]. The heterocellular connection appears to be functional as judged by the passage of calcein orange dye from the tumor cells to the brain endothelial cells, which might exchange other bioactive molecules. Importantly, using 4T-1 breast cancer cells that were labeled with PKH26 membrane dye, which

is retained when labeled cells are not dividing but lost after three cell divisions, the authors showed that the metastatic tumor cells that initiated heterocellular communication with brain endothelial cells may be dormant cells as exemplified by cancer stem cells.

15.16 Hormonalization of Autacoids

Autacoids function in a paracrine fashion in physiological conditions. However, when overexpressed in pathological conditions they appear in the blood stream and affect the distant target cells in an endocrine manner.

Autocrine secretion of VEGF in endothelial homeostasis, that of S100A8 in myeloid cells as exemplified in immune suppressive functions of MDSC and paracrine secretion of TNF α within a variety of tissues are known. Plasma levels of those factors increase in tumor patients in general, which allows their action in tissues distant from the sites of original overproduction, i.e., primary tumors.

For example, S100A8-A9 protein levels in plasma were elevated in gastric cancer patients [114]. Although the S100A8-A9 producing primary tumor is fixed in stomach, the plasma S100A8-A9 works in an endocrine manner to stimulate bone marrow-derived MDSC, which secretes S100A8-A9 forming an autocrine loop. MDSC circulate and migrate to a tissue including the primary tumor and distant ones apart from it, in which the original functions of S100A8-A9 are transmitted.

15.17 Premetastasis Precedes Danger (Damage)

In the danger theory, Matzinger proposed that the immune system is activated by damaged cells (Fig. 15.6) [115]. This led to the molecular identification of damage signals by so-called alarmins or danger-associated molecular patterns (DAMPs), including uric acids, HMGB1 (see Chap. 6), and S100A8 (Chap. 15). Although Matzinger described by mistake that the immune system fails to be activated by tumors, because they usually do not become necrotic to give DAMPs [116], current understanding is that tumors cause tissue damages and therefore premetastasis, which is devoid of tissue damages, precedes the arrival of tumor cells as a true danger.

15.18 Concluding Remarks

Hijacking of homeostatic roles played by endogenous mediators by tumor cells gives inflammatory conditions in premetastatic tissues.

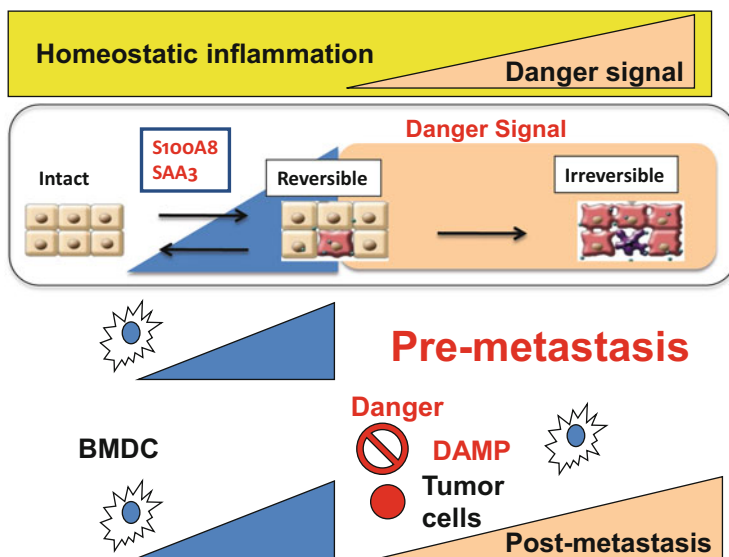


Fig. 15.6 Danger theory and premetastasis

Establishment of premetastatic milieu precedes damages caused by the arrival of metastasizing tumors as a true danger

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Chapter 16

Therapeutic Potential

At the beginning, I need to mention that the drugs described in this chapter are either of practical use in current clinical settings or previously were designed in subclinical situations but I suppose still are valuable to shed light on the translational research in inflammation-associated disorders.

16.1 Examples of Metastatic Tumors

I present two of our cases of metastatic tumor (Fig. 16.1). The first case is a patient with malignant lymphoma who underwent efficient chemotherapy followed by recurrence three times with metastatic progression in liver for the first time, colon for the second, and skin for the third. Each time the metastasis appeared, negative results in imaging studies were achieved by the standard chemotherapy with cytotoxic agents.

The second patient suffers from lung cancer with lymphangitis carcinomatosa. It is thought that lymphangitis is caused by massive hematogenous dissemination of tumor cells that move back into pulmonary lymphatic ducts and is one of the most difficult modes of tumor progression to treat. The patient was efficiently treated with gefitinib with success. Although metastatic lesions are rather difficult to eliminate by chemotherapy, almost perfect regression of tumor cells at the metastatic sites could take place particularly in the initial trials.

As of July 2014, the American Society of Clinical Oncology (ASCO)-guided therapy for advanced breast cancer patients, such as those with brain metastasis, is local therapy, such as surgery, radiation and radiosurgery, and systemic therapy that includes docetaxel, trastuzumab, and pertuzumab in HER2+ cases prone to brain metastasis. This means that even now the main strategy against metastasis in breast cancer is to target tumors by themselves [1].

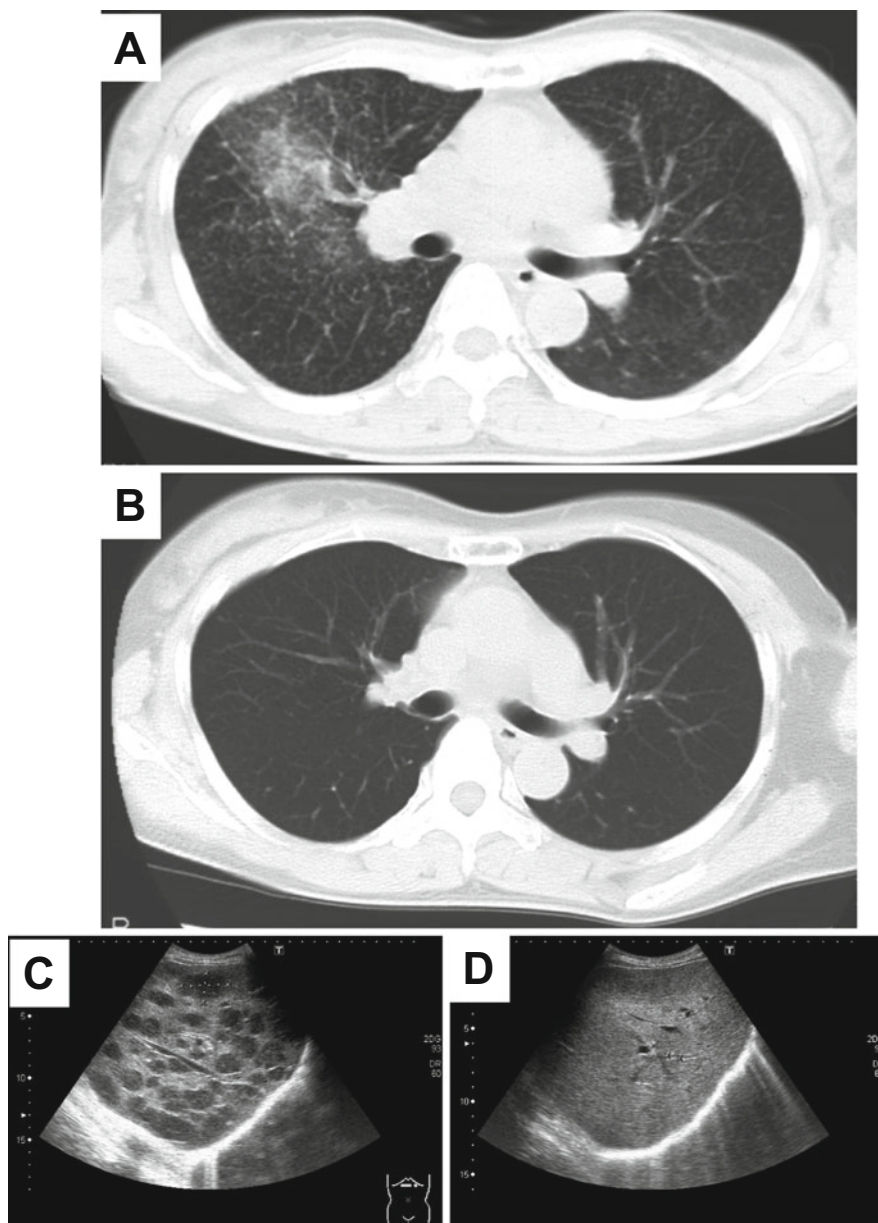


Fig. 16.1 Amelioration of metastasis in liver and lung

Here are two patients: one with non-small lung cancer with pulmonary lymphangitis and the other with malignant lymphoma with liver metastasis

Treatment of the lymphangitis patient (A) aged at 45 with gefitinib resulted in elimination of tumor cells in the lungs (B). However, unfortunately she left the hospital by private reasons and no further information is available. The other patient underwent distal gastrectomy at age 73 years for diffuse B-cell malignant lymphoma that formed 2-cm ulceration in the stomach angle but with no

16.2 Malignant Melanoma as a Model of Anticancer Therapy

In this book, I frequently refer to melanoma cells for experiments, because they are well known for their high metastatic potentials. What are dermatologists doing in clinical settings? I introduce the most updated but still improving practical treatment of melanoma in 2014 from the standpoint of inflammation.

An estimated death rate is roughly 2 in 100,000, similar to that in chronic myeloid leukemia (CML). You can see the usual melanoma in the skin just by looking with your eyes. Time-wise inspection and surgical access are easy for skin lesions in general. Although surgical resection always should be the best way to have complete remission at the levels of current clinical practice, the most prominent feature of malignant melanoma is clinically unrecognizable invasive dissemination of tumor cells even by CT, MRI, and PET.

A classical cytotoxic drug dacarbazine is an alkylating agent approved by the U.S. Food and Drug Administration in 1975 for the treatment of malignant melanoma. It took 10 years for Japan to give permission for the import and clinical application in 1986. It has been effective in some cases of metastatic melanoma. Its metabolite diazomethane CH_2N_2 is involved in the methylation of DNAs. This is a similar circumstance to busulfan, another alkylating agent for CML, which had been long used for the treatment before the advent of molecular-targeted drug imatinib as I described in detail in Chap. 3. Conversely, in melanoma, mutations in BRAF were discovered in 50–60% in Caucasian and roughly 25 % in Chinese and Japanese [2]. One primary site could contain polyclonal mutations, such as the first cell with BRAF V600E, the second cell with BRAF V600K or have double mutations, both BRAF V600E and c-Kit V56G while CTC has only c-Kit V56G. BRAFV600E is not specific to melanoma but found also in 50 % of papillary thyroid cancer and 64 % of histiocytosis X (HX) as described before.

BRAF is upstream of the MEK-ERK cascade in Ras-mediated growth signaling and an orally available selective inhibitor called vemurafenib against BRAF V600E, but not against wild-type BRAF, was developed. A phase 3, randomized, clinical trial to compare dacarbazine with vemurafenib in previously untreated patients of metastatic melanoma was documented in 2011. For example, the calculated hazard ratio in the analysis of progression-free survival (PFS) of 549 patients was 0.25 (95% confidence interval [CI], 0.20–0.33; $P < 0.001$) (see notes below for statistics) with the estimated median PFS of 5.3 months in



Fig. 16.1 (continued) pathological evidence of invasion to the coresected spleen and lymph nodes. Chemotherapy perfectly eliminated the liver metastasis (**D**) that was developed 4 years postoperation (**C**). Another round of chemotherapy was effective for the relapse in the abdominal wall completely disappeared, which developed 2 months after the partial resection of small bowel at which the tumor relapsed for the second time 6 years after the initial operation to cause intestinal obstruction. Rectal invasion, massive ascites with atypical lymphocytes, and severe leukoerythrocytosis appeared 7 years after the third remission and died

vemurafenib compared with that of 1.6 months in dacarbazine [3]. The hazard ratios of postmetastatic stages after hematogenous spread, such as M1b (lung metastasis, $N = 102$) and M1c (extrapulmonary metastasis, $N = 368$), were 0.34 (0.19–0.59) and 0.24 (0.18–0.32), respectively. The hazard ratio of IIIC (regional lymph node involvement, $N = 24$), which could be assumed as premetastatic (see Chap. 13) stage for the lungs, was 0.06 (0.01–0.54).

It is clear that vemurafenib is effective, but resistance appears in half a year on average and the MEK signaling is restored back to the melanoma. The robustness of the RAF-MAPK signaling cascade is controlled homeostatically by the relatively rapid negative feedback by Erk on RAF [4]. If you inhibit MEK located in between, decreased activation in Erk results in reduced negative feedback on RAF, leading to the stronger RAF to MEK signaling with which the inhibitor needs to cope with. Therefore, MEK inhibitors should act more efficiently in cells carrying a mutated BRAF with no such feedback. See signaling scheme in Chap. 10.

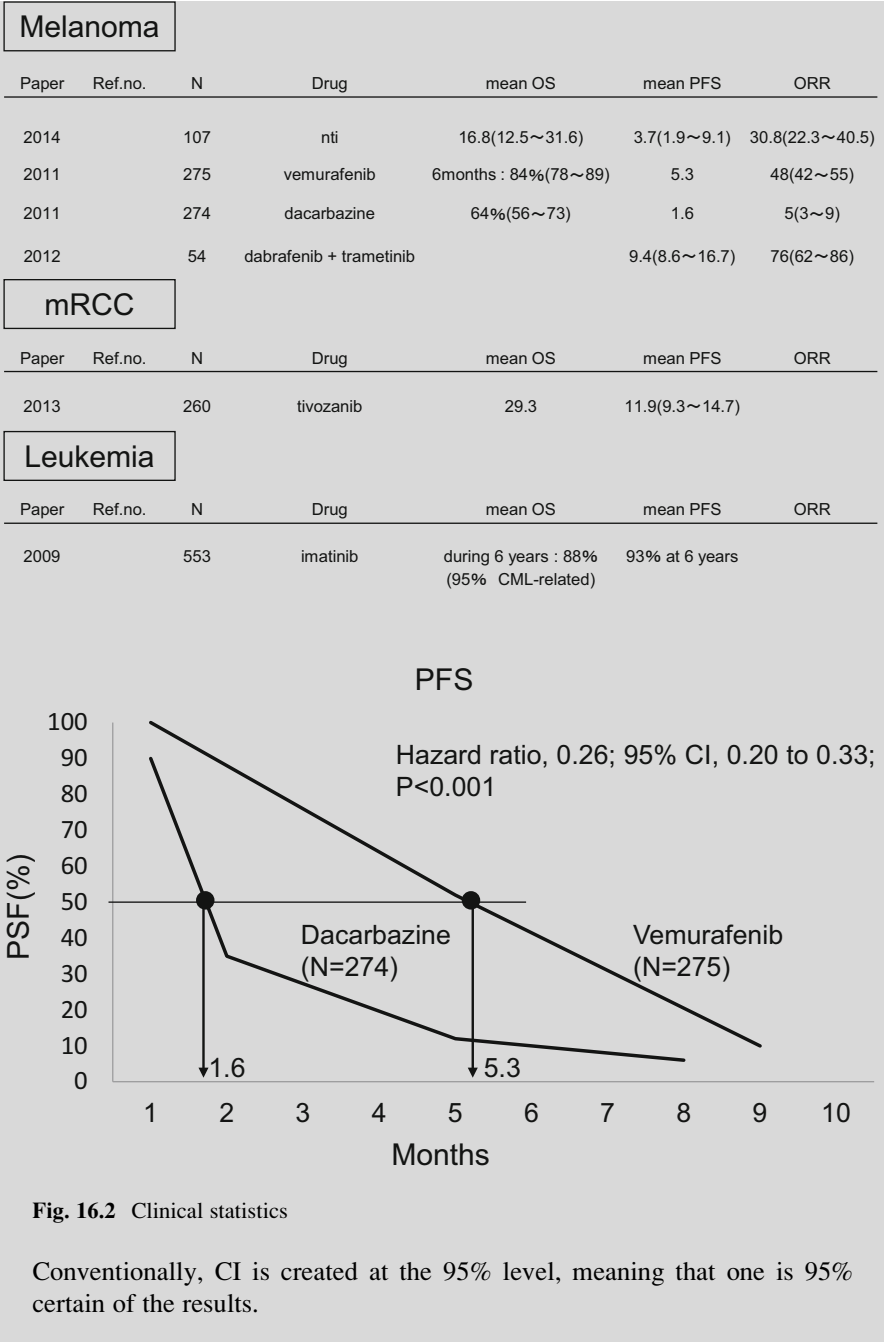
A more highly selective inhibitor for mutated BRAF, called dabrafenib, was developed and put into clinical trial in combination with trametinib, a selective MEK inhibitor with 247 patients of metastatic melanoma [5]. The median PFS was nearly doubled to 9.4 months (hazard ratio for progression or death 0.39; 95% CI, 0.25–0.62, $P < 0.001$). The hazard ratio was 0.28 (0.12–0.66) for 33 patients of the subgroup consisting of IIICM0, IVM1a, or IVM1b and that of IVM1c of 75 patients was 0.45 (0.26–0.78). The objective response rate was 76%.

Clinical Statistics

While overall survival (OS) is defined as the time from the randomization to death of any cause, progression-free survival (PFS) is that to the time of disease progression or death (Fig. 16.2). Hazard ratio, often used as the same meaning as relative risk, is chance of events (melanoma progression, for example under vemurafenib as described above) that takes place in the treatment arm (vemurafenib) compared with that in the control arm (dacarbazine). Therefore, the hazard ratio 0.25 indicates the chance of the disease progression under vemurafenib is 25% of that under dacarbazine at any time. The information related to time, such as median survival time, should be considered for better understanding of the analysis as described < the estimated median PFS of 5.3 months in vemurafenib compared with that of 1.6 months in dacarbazine > [2].

To assess that the apparent therapeutic effect is real or could simply be a chance, the same statistical theory and calculations underlie P value and confidence interval. There is less than a 5% chance that the results arise by chance under P value < 0.05 . $P < 0.05$ and $P < 0.01$ is sometimes considered significant and highly significant, respectively. The CI 0.20–0.33 provides a range usually created in such a way that the range captures the true effect.

(continued)



(continued)

Here is one more example of randomized trial to test if regular sunscreen use is effective against melanoma development. In 1992 through 2006, almost 15 years of observation of randomly selected 1,621 residents of Queensland, Australia, gave hazard ratio of 0.50 (95% CI 0.24–1.02, P value = 0.051) between the sunscreen group (11 melanoma out of 812) and no sunscreen group (22 of 809) [6]. Because the CI captures the value of <no effect> (hazard ratio of 1.0) and $P > 0.05$, this is nonsignificant, the true effect value is anything from 86% reduction to 2% increase in melanoma development. The development of invasive melanoma gave hazard ratio of 0.27 (95% CI 0.08–0.97, $P < 0.045$), indicating 73% decrease with statistical significance.

Melanoma cells actively produce immunological mediators. Melanoma cells secrete VEGF and IL-6 to stimulate DC, which in turn express TNF α and IL-12. A MEK inhibitor U0126 was known to suppress production of VEGF and IL-6 in A375 melanoma cells with BRAFGV600E [7].

An agonistic anti-CD40 antibody that can activate CTL against pancreatic cancer has been described [8]. An antibody-mediated crosslinking of CD40, which belongs to the TNFR superfamily, has been known to condition DC to become an activator for cytotoxic T cells in a manner independent of the presence of MHC class II and produced cytokines, including IL-6 and IL-12. The agonist CD40 antibody in combination with gemcitabine was applied to the KPC model of pancreatic ductal cancer with 30% tumor regression. This could take place in the absence of both CD4⁺ and CD8⁺ cells by antibody-mediated depletion, but clodronate-mediated macrophage deprivation abrogated the effect. F4/80⁺ macrophages expressing CD40 are responsible for the tumor regression. A clinical trial with 21 patients revealed therapeutic efficacy with the median progression-free survival (PFS) of 5.6 months and the median overall survival of 7.4 months compared with 2.3 and 5.7 months, respectively, achieved by gemcitabine alone.

An opposite strategy has been taken in another group of drugs effective against melanoma to target the immune checkpoint system as represented by PD-1 (programmed cell death-1) (CD279) and CTLA4 (cytotoxic T-lymphocyte-associated antigen 4) (CD152) on T cells, whose major activities are found in tumor and lymph nodes, respectively (see Fig. 1.3 in Chap. 1) [9]. A gradual decrease of CD8⁺ T-cell functions under chronic infection and cancer is called T-cell exhaustion and accompanied by sustained expression of multiple inhibitory receptors including PD-1. T-cell receptor (TCR) signaling eventually activates the PI3K-Akt pathway that leads to activation of a variety of molecules, including NF κ B. PD-L1 and B7-1 on tumor cells bind PD-1 and CTLA4, to activate SHP2 and PP2A and thereby give negative feedback on the activation of PI3K and Akt, respectively [9]. VEGF-A, which is abundantly expressed in tumor microenvironment, directly induces PD-1 in CD8⁺ T cells in vitro [10]. Conversely, treatment of CT26 tumor-bearing mice

with anti-VEGF-A antibody reduced expression of PD-1 on intratumoral CD8⁺ T cells.

Intrasplenic injection of B16 melanoma cells causes liver metastasis, which was efficiently abolished in the background of PD-1-KO mice [11]. Given that PD-1-KO T cells were highly activated for proliferation and IFN γ production before they encountered the tumor cells, PD-L1 on APC but not tumor cells is more likely engaged in immune suppression of T cells. Importantly, lung metastasis after intravenous injection of CT26 colon cancer cells was also inhibited in PD-1-KO mice [11]. One of the privileges of CTL is that they can attack tumor cells more efficiently when disseminated rather than accumulated as in the primary site.

Antibody against PD-1 (nivolumab) or CTLA-4 (ipilimumab) has been effective against metastatic melanoma with mean progression-free survival (PFS) of 2.86–3.70 months and mean overall survival (OS) of 10.1–16.8 months [12–14]. The objective response rate was 40% when both were used in combination, while it was roughly 30% with the individual use.

The immune check point strategy is not always effective. Roughly 50% of CD11b⁺ cells from CML patients and CML cell lines, such as K562, were shown to have increased expression of PD-L1 [15]. Sorted T cells from CML patients had no change in IL-2-stimulated growth. Blockade of PD-L1 by a PD-L1 antibody had no effect on T-cell growth in coculture of T cell-depleted leukocytes from CML patients with peripheral blood mononuclear cells from a control subject (see Chap. 12).

Recent evidence supported that T-cell exhaustion still took place in PD-1-KO with infection by chronic lymphocytic choriomeningitis virus [16]. Elevated expression of other inhibitory receptors may explain the phenomenon. The elevated T-cell functions and growth in the acute phase of infection is eventually followed by augmented cytotoxicity and extended tissue distribution of terminally differentiated T cells to bone marrow and liver despite decreased cytokine production. Thus, optimal therapeutic strategies for the PD-1 blockade remain to be established.

16.3 Metastatic Trait of Tumor Cells Per Se

In those successful treatments, the molecular targets are essentially of tumor cell origin and involved in cell growth and immune response. Most importantly, the immune checkpoint inhibitors can be used for patients without BRAF mutations, and given that their mean OS is significantly longer than mean PFS it is likely that the patients may survive with the tumor, i.e., by suppressing lethal metastatic progression.

As I stated earlier, the result that the hazard ratio of IIIC was 0.06 (0.01–0.54) with vemurafenib against dacarbazine suggests that prevention of lung metastasis may be possible to target the driver mutations, i.e., BRAFV600E in melanoma.

I compare the recent melanoma results with those of other malignancies (Fig. 16.2). Metastatic renal cell carcinomas (mRCC) have mutations in VHL,

which eventually leads to HIF-1 α activation with increased VEGF expression and signaling as one of the outcomes. One of the VEGFR tyrosine kinase inhibitors tivozanib that were applied to 260 mRCC patients gave mean OS of 29.3 months and PFS of 11.9 months [17]. Readers should notice that although the values are of similar levels to the melanoma trials, the direct drug target is not tumor cells per se. The most leading molecular-targeted therapy in cancer is for CML (see Chap. 9). Analysis is 553 CML patients under imatinib showed that CML-related OS is 95% and progression-free patients are 93% during the 6 years [18]. Readers also can see that CML cells display CTC from the bone marrow but never grow in the lungs from the beginning, giving the excellent results in the tumor cell-targeted treatment even if serum level of VEGF are elevated. Needless to underline, the best strategy against metastatic progression of tumor is unleashing the tumor-mediated immune suppression by attacking tumor cells by themselves.

A simple question is how metastasizing tumor cells are selected against harsh conditions in different organs from the primary. Negative selection pressure on growing cells might provide tumor cells with stem cell-like properties under duress. Roles of translational arrest in stress granules in lung metastasis by human sarcoma cell xenografts are described in Chap. 13 [19]. Autophagy also supports cell survival under metabolic stress in a variety of cells including cancer stem cells [20] (see Chap. 10 for autophagy). More activated autophagy, as monitored by LC3 expression for autophagosome marker, was observed in patient tissue samples from lung metastasis than primary tumor, although it was not specific to lung metastasis. This also was true in a mouse lung metastasis model of hepatocellular carcinoma [21]. Conversely, inhibition of autophagy by shRNA-mediated knockdown of ATG5 and BECN1 suppressed the lung metastasis in the same model [22]. This suggests that strategy for triage in noxious microenvironments can enhance tumor progression.

As I stated earlier in Chaps. 11 and 13, a high efficiency of lung metastasis was observed in an example of CD90⁺CD24⁺ CSC in the MMTV-PyMT mouse breast cancer model [23]. This model revealed an interaction between CSC and lung fibroblasts in which fibroblast-produced extracellular matrix (ECM) protein periostin actively maintains the CSC through Wnt signaling. Metastatic CSC also can protect themselves against immune surveillance. In a murine prostate cancer model, both NK and cytotoxic T cells target the CSC but CSC-overexpressed ECM Tenascin-C suppresses those cells by binding to their $\alpha 5 \beta 1$ integrin [24].

As described in Chap. 11, progressor mutations in metastatic sites also are found in the primary tumors. In addition to genetic alterations, gene expression profiling in cells capable of metastasizing to a given organ has been intensively executed in the Joan Massague group. For example, patient-derived breast cancer cells from pleural effusion and the MDA-MB-231 cell line were subjected to *in vivo* selection for brain metastasis after intracardiac injection to facilitate brain metastasis. By gene expression profiling between the selected cell population and the original they uncovered COX2, HBEGF, ANGPTL4, and ST6GALNAC5. Both COX2 and HBEGF also facilitated extravasation in the lungs. Those gene products are engaged in transendothelial migration of tumor cells by changing the cell-cell

interactions. Generally, ST6GALNAC5, a sialyl-transferase, has restricted expression in brain [25]. Brain-specific expression is hijacked by tumor cells to give brain-specific metastasis.

Then what promotes the expression of those genes? For example, ANGPTL4 was induced by both HIF-1 and TGF β -Smad signaling in breast cancer cells and could increase permeability in HUVEC monolayers as judged by TER, which can facilitate extravasation of the intravenously injected tumor cells in the lungs [26, 27]. The authors reversed both of the *in vitro* and *in vivo* effects by an shRNA-mediated knockdown of HIF-1. TGF β is induced in tumor cells and stromal myeloid cells by hypoxia and TGF β can also induce ANGPTL4, which disrupted endothelial cell-cell junctions.

Then how to understand and cope with the complicated HIF-1 signaling that takes place not only in tumor tissues but also in a variety of human diseases including diabetes mellitus, cardiovascular disease, and obesity? I believe that one such idea is nicely proposed by a review by Matteo Russo [28]. The point is that hypoxia induces not only necrosis to passively release intracellular substances some of which serve as alarmins but also actively promotes expression of their receptors, such as RAGE, P2X7, and TLRs, in the still living cells in the same microenvironment to transmit signals mainly by NF κ B responsible for expression of more than 500 genes. This sequential event appears to occur rather early. Therefore, an early control should be performed for the targets, including (1) HIF-1 activity by, for example, 2ME2 (2 methoxyestradiol) capable of blocking nuclear translocation of HIF-1, or resveratrol, (2) RAGE by aminoguanidine and metformin, etc., (3) P2X7 by its antagonists, and (4) NF κ B by curcumin and bortezomib etc.

The Joan Massague group also uncovered a tumor trait in bone metastasis by breast cancer, which, as I stated in Chap. 9, could have a long latency up to 25 years. They analyzed a cohort of 615 breast cancer patients and found that ER⁺ patients had a long disease-free period in the first 1.7 years but a higher rate of relapse after 5 years after diagnosis especially in bone [29]. The gene expression pattern due to Src activation was found in 88.4% of ER⁺ patients versus 23.0% in ER⁻. Under their similar *in vivo* selection strategy through bone, they established MDA-MB-231 (ER⁻HER2⁻)-derived BoM⁻1833 cells in which Src was constitutively activated as monitored by phosphorylated tyrosine at 416 and bone metastasis took place in 100% to bone within 70 days after intracardiac injection into athymic mice. The bone metastasis was blocked by shRNA-mediated knockdown of Src, but not Fyn and Yes, and by dasatinib, an inhibitor of Src family tyrosine kinases. *In vitro* studies with BoM-1833 cells showed that CXCL12-stimulated activation of Akt counteracted against cell death-inducing activity of TRAIL (Fig. 8.3 in Chap. 8) in a manner dependent on Src. Most importantly, another established ER⁻ cell line CN34⁻BoM2 with activated Src and introduced markers, GFP and puromycin-resistant gene, stayed in bone for 35 days as judged by the markers also in a Src-dependent fashion. One interpretation would be that CXCL12⁻CXCR4 homeostatic signaling pertinent to HSC in bone marrow is hijacked by tumor cells

expressing CXCR4 and activated Src, which escapes the TRAIL-mediated immunological elimination.

Another hijacking is VACM-1 expression in human breast cancer cells in the metastatic lungs but not brains [30]. As shown in Fig. 1.2 in Chap. 1 and Fig. 11.4 in Chap. 11, endothelial VCAM-1 binds its counter-receptor leukocyte $\alpha 4 \beta 1$ (VLA-4) integrin in homeostatic leukocyte trafficking. After extravasation in the lungs, VACM-1 in lung metastatic cell line MDA231-LM2 cells upon engagement with the counter-receptor in macrophages activates Akt to again protect themselves against apoptosis-inducing activity of TRAIL expressed in the metastatic lungs. Do we need to take anti-Src or anti-Akt medicines against metastatic breast cancer after surgical removal of the primary tumor during the whole life?

16.4 Triangle-Based Idea to Treat Tumors

I described an idea of triangle consisting of angiogenesis, coagulation, and inflammation with tumor cells in the center (see Fig. 3.6 in Chap. 3 and Fig. 13.5 in Chap. 13). Conceptually, the triangle is firm in the presence of tumor cells but assumed to be fragile in premetastasis that actually lacks them (Fig. 16.3; Table 16.1).

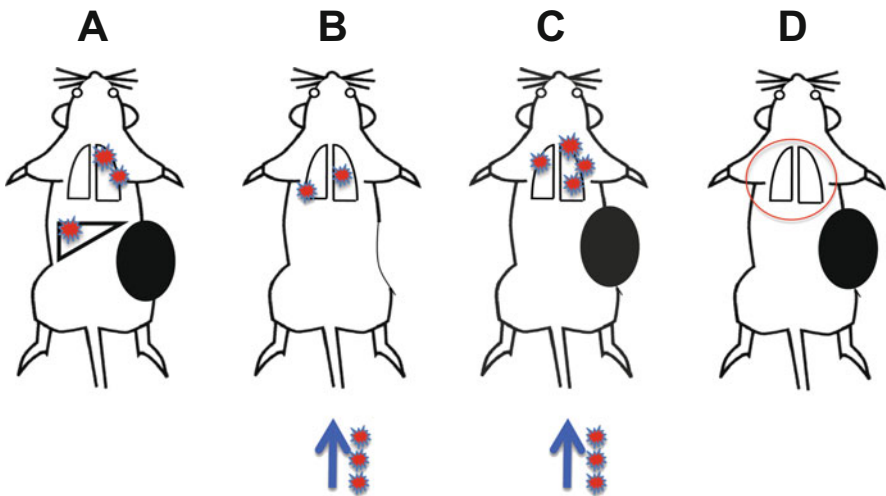


Fig. 16.3 Murine metastasis models

This figure shows the mode by which to evaluate antimetastasis strategies listed in the associated Table 16.1

(A) Implanted tumor cells disseminate to multiple organs such as lungs and liver

(B) Injection of labeled tumor cells through veins (usually the tail vein) into systemic circulation in tumor-non-bearing mice causes lung metastasis

(C) The same injection as (B) in tumor-bearing mice causes lung metastasis more efficiently than that in (B)

(D) Premetastatic lungs that implanted tumor cells never reach

Table 16.1 Necessary conditions for metastasis

Necessary conditions						
	Biology	Targets	Method	Tumor cell	Systems	Primary
1	A-1	VEGF	Antibody	HM7	B(liver)↓	–
2	A-2	VEGFR1	TK-KO	B16	B(lung)↓	–
3	A-3	PIGF	Antibody	B16	B(lung)↓	–
4	A-4	VEGFR1	shRNA	B16-mCherry	B(lung)↓	–
5	A-5	VEGFR2	Inhibitor	RIP-Tag2	A(liver)↑	↑
6	A-6	plasma Fn	KO	B16, 3LL	B(lung)↓	–
7	A-7	Neuropilin-2	Antibody	66c14 mammary carcinoma	A(lung)↓	→
8	A-8	EphA2	KO	LLC	B(lung)↓	–
9	A-9	Angiopoietin-2	Antibody	4 T1	B(lung)↓	–
10	A-10	MMP-9	KO	17L3C-luc	B (lung)↓	–
11	A-11	MMP-13	KO	B16F1-GFP	B (lung, liver)↓	–
12	A-12	ADAM8	Antibody	MDA-MB-231	A (Lung, Brain)↓	↓
13	A-13	Tenascin-C	KO	RT2 insulinoma	A (lung↓, liver→)	↓
14	A-14	Integrin α4	Knock-in (inactive)	PyMT-MMTV	A(lung, lymph node)↓	→
15	A-15	Adp2/3	KO	PC-3 luc	A(lymph node)↓	↓
16	A-16	VCAM-1	Antibody	4 T1	B (lung)↓	–
17	C-1	III (tissue factor)	KO	EF(III-KO)	B(lung)↓	–
18	C-2	III-VIIa	Antibody	CHO/TF(III)	B(lung)↓	–
19	C-3	Plasminogen	KO	LLC, B16	B(lung)↓	–
20	C-4	XIIIa	KO	LLC-GFP	A, B (lung)↓	→
21	C-5	Xa	Inhibitor	L-CI.5S	B(lung, liver)↓	–
22	C-6	II (thrombin)	Inhibitor	B16	B(lung)↓	–
23	C-7	I (fibrinogen)	KO	LLC, B16	A, B (lung)↓	→

(continued)

Table 16.1 (continued)

Necessary conditions						
Biology	Targets	Method	Tumor cell	Systems	Primary	Ref.
24 C-8	PAI-1	KO	B16-BL16	B (lung)→	–	[53]
25 P-1	Nf-E2	KO	B16	B(lung)↓	–	[54]
26 P-2	Par4	KO	B16	B(lung)↓	–	[54]
27 P-3	Par1 or 2	KO	B16	B(lung)→	–	[54]
28 P-4	GPIb	KO	B16	B(lung)↓	–	[55]
29 P-5	GPIIb/IIIa	Inhibitor	LLC	B(lung)↓	–	[56]
30 P-6	Ga α	KO	LLC-GFP	A, B (lung)↓	→	[57]
31 P-7	Platelet, gp44	Antibody	NL-17	B(lung)↓	–	[58]
32 P-8	Oral1	Inhibitor	MDA-MB-231	B(lung)↓	–	[59]
33 P-9	P-selectin	KO, heparin	LS180	B(lung)↓	–	[60]
34 I-1	TLR4	KO	LLC, 3LL	A,B,C (Lung)↓	→(A)-(B)	[61]
35 I-2	TNF α	KO	LLC	B (lung)↓	–	[62]
36 I-3	TLR2	KO	LLC-DsRed, LLC	B (lung)↓, A (lung, liver, adrenal)↓	→(A)-(B)	[62]
37 I-4	COX	Inhibitor	410.4	A, B (lung)↓	↓	[63]
38 I-5	HGF	HGF antagonist	B16F10, LLC	B (lung)↓	–	[64]
39 I-6	HGF	HGF antagonist	MC38	B (liver)↓	–	[65]
40 I-7	TGFb	Inhibitor, neutralizing Ab	4 T1, MDA-MB-231	A (lung)↓	↓	[66]
41 I-8	TGFb	TGFbR inhibitor	MDA-MB-231	A, B (lung, bone)↓	↓(A)-(B)	[67]
42 I-9	G-CSF	Neutralizing Ab	4 T1, MDA-MB-231, PyMT	A (lung)↓	→	[68]
43 I-10	MMP-8	KO	B16F10, LLC	A, B (lung)↑	→	[69]
44 I-11	MMP-13	KO	E0771	A (lung)↑	→	[70]
45 I-12	AhR	KO	B16F10	B(lung)↓	–	[71]
46 I-13	Hsp70	KO	PymT	A (lung)↓	↓	[72]

47	I-14	EP4	Inhibitor	66.1	B (lung)↓	—	[73]
48	I-15	ADAM12	KO	PyMT-driven mammary adenocarcinoma	A (Lung)↓	↓	[74]
49	I-16	Cbl-b	KO	TC-1, B16F10	A B (lung)↓	↓	[75]
50	I-17	TLR7	KO	LLC	B (lung)↓	—	[76]
51	I-18	Periostin	KO	MMTV/PyMT	A(lung)↓	→	[23]
52	I-19	MD-2	KO	E0771	B (lung)↓	—	[77]
53	I-20	Ccl5	Inhibitor	MC38, LS180	B (lung)↓	—	[78]
54	I-21	SAA3	Antibody	LLC	C (lung)↓	—	[61]
55	I-22	CXCR4	KO (+/—)	B16	B (lung)↓	—	[79]
56	I-23	TAM receptor	Inhibitor	B16F10, 4 T1	A(liver)↓, B (lung)↓	→	[75]
57	T-1	ADAM15	shRNA	PC-3	B (Bone, Lung)↓	—	[80]
58	T-2	EGFR	EGFR inhibitor	SUM149	A (lung)↓	↓	[81]
59	T-3	AhR	shRNA	MDA-MB-231	B(lung)↓	—	[82]
60	T-4	Plasma Hsp90a	Antibody	B16/F10, MDA-MB-231	A (lymph nodes, lung) liver↓	→	[83]
61	T-5	ZEB1	shRNA	HCT116	A(liver)↓	→	[84]
62	T-6	Fra1	shRNA	MDA-MB-231	B (lung)↓	—	[85]
63	T-7	BACH1	shRNA	MDA-MB-231	B (Bone)↓	—	[86]
64	T-8	Cathepsin S	shRNA	MDA-MB-231-TK-GFP-Luc	A (Brain)↓	→	[87]
65	T-9	ELF5	Overexpression	4 T1, MDA-MB-231	A, B (lung)↓	-(B), →(A)	[88]
66	T-10	CoCo	shRNA	MDA-MB-231	B (lung)↓	—	[89]
67	T-11	CEBPδ	KO	MMTV-Neu mammary tumor	A (lung)↓	↑	[90]

(continued)

Table 16.1 (continued)

Necessary conditions							
	Biology	Targets	Method	Tumor cell	Systems	Primary	Ref.
68	T-12	LOX	shRNA	MDA-MB-231	A (lung)↓	ND	[91]
69	T-13	CXCL1	shRNA	LS174T	B (liver)↓	–	[92]

Well-known factors in the host arm, including angiogenesis (A), platelet (P), coagulation (C), and inflammation (I) as well as in the tumor cell arm (T) are targeted by antibody, shRNAs, genetic knockout, or pharmacological inhibition. The influences on the primary tumor and metastasis as evaluated by experimental methods in Fig. 16.3 are shown. It cannot be distinguished perfectly whether a given factor is related to A or T. For example, VEGF is produced from both macrophages of host origin and tumor cells. Anti-VEGF antibody inhibits liver metastasis of HM7 colon cancer cells after splenic-portal injection without establishing the primary tumor (A-1). Needless to say, VEGF of both origins is inhibited. On the other hand, embryonic fibroblasts (EF) (C-1) derived from tissue factor (coagulation factor III)-KO mice were transformed by an oncogene and subjected to the type B metastasis assay. An effect on the III-stimulated activation of host coagulation system was negative in lung metastasis. While B16 melanoma cells displayed poor lung metastasis in Ahr-KO mice (I-12), an shRNA-mediated knockdown of Ahr in MDA-MB-231 tumor cells (T-3) cells inhibited their lung metastasis after intravenous injection without the primary tumor. TK-KO (A-2); Tyrosine kinase knockout, Fn (A-6); Fibronectin. Furthermore, effects may vary in a context-dependent manner. For example, PAI-1-KO mice failed to affect the primary tumor growth and lung metastasis also in the MMTV-PyMT breast cancer model [93]. Effects of PAI-1-KO or PAI-1 overexpression by transgene on tumor invasion and angiogenesis of subcutaneously implanted malignant murine keratinocytes PDVA cells showed a bell-shape effect of PAI-1 [94]. Both absence and excess PAI-1 resulted in tumor reduction

16.4.1 *Target Angiogenesis*

I have discussed on the effective but still limited results of inhibitors of VEGFR tyrosine kinases in cancer as represented by metastatic renal cell carcinoma patients.

16.4.1.1 VEGFR1

Vascular mimicry (see Chaps. 11 and 13) is a combined phenomenon of metastatic tumor cell trait and angiogenesis, and it could be an interesting therapeutic target since malignant melanoma-initiating cells (MMIC) with repopulating ability supports CD31⁺VE-cadherin (CD144)⁺TIE1⁺ laminin⁺ vascular mimicry [95]. Preferential expression of the ATP-binding cassette member (ABCB5) is observed in MMIC and melanoma CTCs are ABCB5⁺. In addition, a subpopulation of ABCB5⁺ cells was found to express VEGFR1 in clinical samples [96]. VEGF could induce CD144 expression in ABCB5⁺ cells, which was abrogated by anti-VEGFR1 antibody from 42.5 to 1.8%. In a subcutaneous xenograft model, although ABCB5⁺ VEGFR1⁺ cells initiated tumor cell growth they lacked tumor cell-derived laminin expression. An shRNA-mediated knockdown of VEGFR1 in human melanoma cells inhibited vascular mimicry by 86%.

16.4.1.2 PIGF

Anti-PIGF inhibitory antibody, which was capable of inhibiting both VEGFR1 and NP-1, failed to reduce tumor angiogenesis and tumor volume in the primary tumor [32]. PIGF-stimulated proliferation of 67NR-VEGFR1 cells was weak but still significant, which was also suppressed by the antibody. Of note, we have shown that tumor growth of PIGF-overexpressing LLC was less efficient in VEGFR1-tk-KO mice and anti-PIGF antibody inhibited lung colonization of intravenously injected B16F10 melanoma cells [97, 98]. The lodging suppression by NP-2 blockade may involve inflammation-associated mechanisms that remain uncovered [36].

Instead of targeting the frontline cytokines in angiogenesis, I review the events before their production. The abnormal vasculature with hypoxic responses form a vicious cycle, i.e., hypoxic transcription factor HIF-1 α -induced VEGF aggravates the abnormal vasculature, which in turn enhances hypoxia. The oxygen sensor molecule PHD2 controls the HIF-1 α hydroxylation and subsequent degradation. PHD2 by itself is controlled by HIF-1 α . In hypoxic tumor microenvironment ROS production and low pH take place as described in details in Chap. 11. PHD2 is inactivated by oxidation by ROS allowing stability of HIF-1 α . NAC and vitamin C could inhibit ROS thereby normalizing the HIF-1 α status in addition to the suppression ROS-mediated genomic instability [99]. This was demonstrated by the

conditional expression of Myc, which ultimately causes ROS, in human P493 B cell model. NAC diminished HIF-1 α levels, which could be rescued by the expression of mutationally stabilized HIF-1 CA5.

16.4.1.3 PHD2

An interesting phenomenon has been documented by Peter Carmeliet group in 2009 [100]. In PHD2 heterozygously deficient mice, both HIF-1 α and HIF-2 α were up-regulated as expected due to the insufficient negative control by PHD2. HIF-1 α -dependent overexpression of genes responsible for tumor vasculature as represented by VEGF failed to aggravate the situation but instead HIF-2 α -dependent overexpression of a soluble form of VEGFR1 (sVEGFR1) capable of giving negative effects on VEGFR2 signaling and that of VE-cadherin that strengthen the endothelial barrier, both contributed to counterbalance the VEGF-mediated aggravation to normalize the tumor vasculature. Restoration of vessel maturation and endothelial lining with reduced necrotic and hypoxic areas as judged by pimonidazole staining without affecting tumor vessel density and lumen size was observed in ectopic LLC and orthotopic Panc02 tumor models. It was accompanied by approximately 50–70% reduction in the frequency of lung or liver metastasis. Studies with endothelial cell-specific conditional PHD2 haplo-deficient mice also supported the results (Fig. 16.4). Those experiments make us suppose that anti-PHD2 drugs, if any, might be effective against metastatic progression by tumor vessel normalization.

16.4.1.4 Sema3A

Another potentially valuable molecule that may normalize the tumor vasculature is semaphoring 3A (Sema3A) (see Chap. 11). In a spontaneous mouse model of pancreatic neuroendocrine tumor RIP-Tag2 (see Chap. 12), adeno-associated virus-8-mediated expression of Sema3A increased the number of NG2+ pericyte coverage, reduced the vascular density and branching, hampered hypoxia as monitored by pimonidazole staining, down-regulated HIF-1 α -dependent expression of carbonic anhydrase 9 and Met, inhibited Snail expression with increased levels of E-cadherin, increased vessel perfusion as judged by FITC-lectin and inhibited vascular permeability of FITC-dextran [101]. The normalization of tumor vessels and cancellation of EMT in tumor cells were further supported by HPV16/E2 mouse model (see Chap. 12) of cervical papillomas. Sema3A expression reduced by incidence both lung and liver metastasis to roughly 10% from more than 50% also by diminishing the primary tumor growth by 50%. In addition to the effect of Sema3A alone, Sema3A also normalized the tumor vasculature that was aggravated by sunitinib in those mouse models, a VEGFR2 inhibitor. Given that Sema3A antagonizes with VEGF in endothelial cell growth but enhances VEGF activity

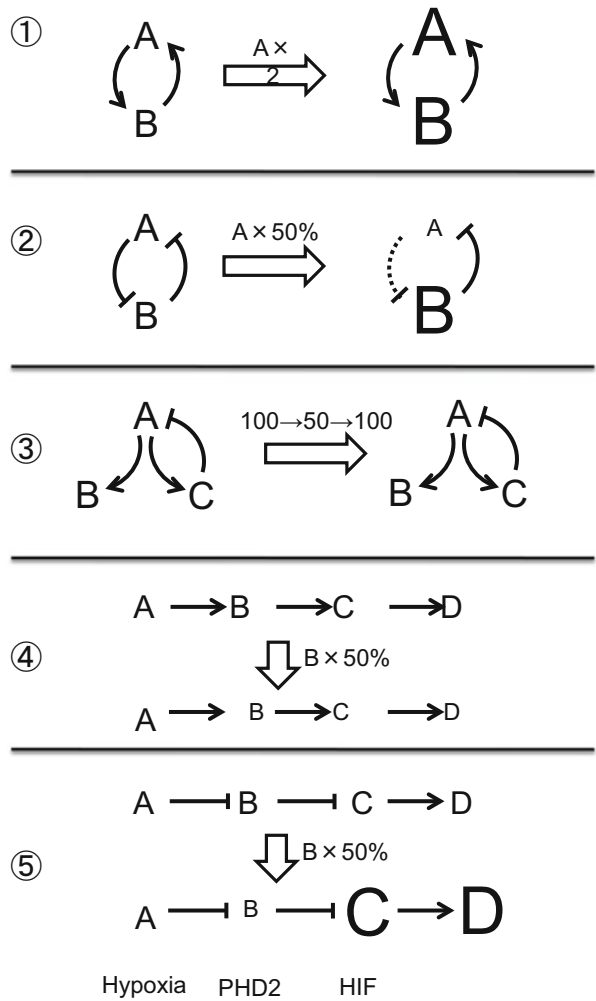


Fig. 16.4 Logics in signaling

When the signal intensity of A for B is enhanced by twofold in a reciprocally feed forward relationship between A and B, both A and B are augmented (1). If the signaling is reciprocally negative and the intensity from A is reduced by 50%, A is diminished while B is augmented (2). Under the condition where A elicits positive signaling for both B and C, and one of them, C for example, is inhibitory against A, B can be neither augmented nor reduced when A is reduced by 50%. Because the reduced A signal for C diminishes the inhibitory C signal against A for reactivation to 100% (3). When B is reduced by 50% in a sequence of signaling from A through D, both C and D downstream of B are reduced (4). In a circumstance in which hypoxia (A) inhibits PHD2 (B) capable of suppressing HIF (C), reduction of B by 50%, by for example haplotypic deficiency in gene knockout, both C (HIF) and D (HIF targets of transcription) are augmented (5)

for vascular permeability, the precise mechanisms of reshaping the abnormal tumor vessels by conferring plasticity on them still remain to be elucidated.

16.4.2 Target Coagulation

Gabriel J. Gasic showed the biological significance of platelet in tumor metastasis experiments in 1968 [102]. Intraperitoneal implantation of TA3 ascites tumor cells exhibited lung metastasis in 2 weeks, which was reduced by 65% under the condition in which antiplatelet serum decreased the platelet count to 3.4% of the initial level in 24 h. It was later confirmed that anti-VWF antibody inhibited the lung metastasis by roughly 50% that was induced by intravenous injection of CT26 colon cancer cells or B16a amelanotic melanoma cells without decreasing the platelet counts while negative results were obtained by antiplatelet agent aspirin capable inhibiting COX for production of both PGE2 and PGI2 [103].

Inhibition of experimental metastasis by genetic or pharmacological blockade of SOCE is described in Chap. 12.

Before I discuss S100A8, an endogenous TLR4 ligand, I will describe an effective treatment in endotoxemia in which LPS plays a major role at least initially. As stated in the triangle idea in Chap. 3, thrombin (as coagulation) is upstream of activated DC response (as inflammation) in sepsis. Recombinant human activated protein C (rhAPC) initially improved survival in septic patients [104]. Infusion of rhAPC reduced leukocyte accumulation in the air spaces of volunteers who had been subjected to LPS instillation as sepsis-induced organ dysfunction. In a murine model of lethal sepsis, APC was ineffective when bone marrow cells were deficient in endothelial cell protein C receptor (EPCR) [105]. It turned out that EPCR on DC was responsible for the efficacy. In addition to EPCR, the improved survival by the treatment by APC could be mediated by CD11b in macrophages and/or $\beta 3$ integrin in neutrophils. However, the drug APC was withdrawn from the market 10 years after approval by FDA, because it ultimately failed to improve mortality rate of patients in severe sepsis [106].

Thrombomodulin (TM) (see Chap. 3) is an antithrombotic transmembrane protein expressed on the surface of endothelial cells with the extracellular lectin-like domain capable of binding to HMGB1 (see below) [107] in the nanomolar range as judged by surface plasmon resonance. The binding neutralizes HMGB1 activity. In addition, another extracellular region called the EGF-like domain binds thrombin thereby activating APC. The TM-bound thrombin cleaves HGMB1 further inactivating its activity. In UV-irradiated inflammation in the mouse skin at 254-nm length (UVC less than 280 compared with the range 280–315 nm in UVB), both anti-HMGB1 antibody and recombinant human soluble form of TM reduced leukocyte infiltration, local TNF α accumulation, and skin swelling by more than 50% [99, 107].

16.4.2.1 Fibrinogen

We have observed abundant fibrinogen deposits in the lungs of patients who died of cancer that were assumed to be premetastatic lungs but not in those by accident [77]. It has been known for a long time that S100A8-stimulated neutrophils acquired activated Mac-1 ($\text{CD11b}^+\text{CD18} = \alpha\text{M}-\beta 2$) but not $\alpha\text{v}\beta 3$, which is responsible for adhesion to fibrinogen.

Subcutaneously implanted B16 and LLC tumor cells could grow in the background of genetic fibrinogen-KO. However, when those tumor cells were intravenously injected via the tail vein, the fibrinogen-KO condition resulted in reduced lung metastasis by roughly 20-fold compared with wild-type mice, which was interestingly enhanced by a naturally occurring peptide-type specific thrombin inhibitor, hirudin derived from leeches and capable of binding to the enzymatic active center of thrombin in circulation and bound to fibrin [48]. Although the negative effects by hirudin is small, because those by fibrinogen-KO was so dramatic. This suggests fibrinogen-independent functions of thrombin that contribute to lung metastasis.

Then what is the mechanism? S100A8-activated myeloid cells bind fibrinogen through CD11b presumably before and during establishment of premetastatic microenvironment in the lungs. Fibrin is observed in the premetastatic lungs. Both fibrin and fibrinogen are degraded by plasmin. If the presence of fibrinogen by itself supports metastasis, it may be enhanced by plasminogen-KO. However, it was not the case. Recruitment of intravenously injected radiolabeled LLC cells in the lungs of fibrinogen-KO mice was 75% reduced at 24 h after injection. KO mice of fibrin-stabilizing factor XIII abrogated lung metastasis in the forced metastasis model by intravenous injection of B16 or LLC, or spontaneous metastasis in the lung and liver after surgical resection of the subcutaneous primary tumor although the growth of the primary tumor and, intriguingly, lymph node metastasis were unaltered [49]. Stabilization of fibrin is likely against NK cells, because the inhibitory effect for metastasis was shown to be dependent on the presence of NK cells by the NK-cell depletion experiments by anti-asialo GM1 antibody.

In addition to the protective role by fibrin in hematogenously disseminated tumor cells against innate immune cells, fibrinogen was claimed to be a functional TLR4 ligand back in 2001 [108]. They described that <In the experiments reported here, culture medium contained less than 1 EU/ml endotoxin as determined by LAL assay> (see Chap. 7), which is too high to exclude LPS contamination, although they tried to logically rule out LPS contamination by showing sensitivity of the fibrinogen preparation to boiling and C3H/HeJ TLR4 mutation background, and resistance of LPS-induced CCL2 induction by their fibrinogen that might block LPS. Blockade of thrombin activity present in the tissue culture medium by hirudin failed to change the CCL2 induction by the fibrinogen, claiming that fibrinogen to fibrin conversion was not required for the effects. More recent experiments in 2008 to prove the point showed that HEK293 $\text{CD14}^+\text{MD2}$ cells transfected with TLR4 secreted CCL2, IL-6, and $\text{TNF}\alpha$ in response to fibrinogen whose endotoxin

contamination levels in tissue culture medium were they claimed less than 0.005 EU/ml [109].

Moreover, in bone marrow-derived macrophage (BMDM) cultures with fungus *Aspergillus niger* to evaluate their inhibitory effects on fungal growth in the presence of fibrinogen or thrombin-cleaved products of fibrinogen (FCP), FCP but not fibrinogen showed the anti-fungal activity (see Chap. 7) [110]. They claimed that FCP was not involved in clotting without showing its structural details, but FCP induced expression of IL-13R α 1 in both BMDM and airway epithelial cells in a TLR4-dependent manner. Although physical and biochemical evidence is missing in this allergic airway model, FCP but not fibrinogen per se appears to be a functional endogenous ligand for TLR4.

Considering the recent finding that transformed self β -amyloid directly binds and oligomerizes fibrinogen, which not only becomes resistant to thrombin but also integrate the β -amyloid in the oligomer in vascular-related Alzheimer's disease, we may need to think of nonclotting function of fibrinogen and FCP also in tumor biology [111, 112].

16.4.2.2 16 K PRL

The aminoterminal fragment of prolactin (16 K PRL), but not full-length PRL, was shown to bind PAI-1 with K_d of 1.1 μ M and inhibit its activity [113]. PAI-1 is a fibrinolytic inhibitor promoting coagulation (Fig. 3.5 in Chap. 3) and angiogenesis-mediated tumor growth. 16 K PRL not only protected mice against systemic thromboembolism induced by injection of thromboplastin, but also inhibited B16F10 melanoma growth in a manner dependent on PAI-1. Mechanistically, PAI-1 was shown to bind to the PAI-1-uPA-UPAR complex thereby impairing the suppressing effect of PAI-1 on uPA.

16.4.2.3 Warfarin-TAM Receptor

The well-known oral anticoagulant warfarin has been long used in clinical settings since its discovery in 1943 under strict therapeutic dose monitoring. Needless to say, overdosed application may cause serious side effects by lethal hemorrhage. Vitamin K-dependent coagulation factors II, VII, IX, and X, are activated by γ -carboxylation of their glutamate residues. The concomitantly generated vitamin K epoxide is recycled back to a reduced form of vitamin K by vitamin K epoxide reductase, the target of warfarin. Thus warfarin inhibits γ -carboxylation-dependent activation of those coagulation factors indirectly through the regeneration block of reduced vitamin K. Tumor metastasis was reported to be less frequent in patients under warfarinization [114], which is seemingly consistent with the idea that coagulation facilitates tumor progression as discussed elsewhere in this book. The vitamin K-dependent γ -carboxylation also can take place in Gas6, which impairs its ability to activate TAM receptor tyrosine kinases TYRO3, XAXL, and MER

[115]. Those receptors suppress inflammation in a manner mediated by SOCS (suppressor of cytokine signaling) in DC and macrophages. For example, pretreatment of DC by Gas6 inhibits LPS-induced TLR4 signaling by SOCS1 and SOCS3 that are induced by the TAM receptor-Stat1 pathway [116]. Therefore, warfarin can abrogate the negative feedback against TLR activation, in other words, appears to facilitate innate immunity.

The E3 ligase Cbl-b ubiquitinates TAM receptors [75]. Cbl-b-KO had no effect on NK-cell development but both Cbl-b-KO and a highly selective TAM tyrosine kinase inhibitor LDC1267 conferred resistance on the Gas6-induced suppression of growth and IFN γ production in NKG2D-activated NK cells. Crossing Cbl-b-KO mice with NeuT⁺ transgenic mice of breast cancer model that spontaneously develop metastasis showed reduction in primary tumor growth and accumulation of NK cells within the metastatic foci whose number was comparable to that in the control. LDC1267 treatment markedly reduced metastatic progression of intravenously injected B16F10 melanoma cells, which was dependent on the presence of NK cells. They also confirmed that low dosages of warfarin gave the similar anti-metastatic effects on B16F10 without affecting coagulation.

The negative feedback on TLR4 signaling is known to be induced by CD11b. CD11b-activated Syk (see below) tyrosine-phosphorylates MyD88 and TRIF that are subjected to ubiquitination and degradation by Cbl-b [117].

Whereas LPS- and S100A8-activated TLR4 promotes metastasis (see below and Chap. 12), inhibition of its negative regulator TAM, i.e., positive regulation of TLR4 suppresses metastasis. The levels of biological effects may differ in the de-inhibition. S100A8 up-regulation may evade the negative feedback due to its low levels. The efficacy of deinhibition may be large enough to exert beneficial consequences in NK cells as in the case of PDL-1 antibody therapy in melanoma patients (see above).

16.4.3 Target Inflammation

16.4.3.1 Lipid Mediators

PGE2

Tumors formed by LLC that expressed IL-1 β had CCL2-dependent infiltration of macrophages expressing COX2, which reminds us of COX2 expression in breast cancer cells capable of extravasation in the lungs and brain [118]. Higher the COX2 expression levels, breast cancer patients are more prone to have metastasis [119].

The PGE2-EP4 system also contributes to bone resorption. I discussed the RANK-RANKL system in the Chap. 15. During bone resorption, cytokines, such as TGF β , that promote tumor growth are released resulting in a vicious cycle in the postmetastatic bone. Anti-RANKL antibody, called denosumab, is now used in clinical settings in which bone resorption is active, such as osteoporosis and bone

metastasis. To state on the clinical result first, the median bone metastasis-free survival of 716 denosumab-treated patients of nonmetastatic castration-resistant prostate cancer was 29.5 months with hazard ratio 0.85 [0.73–0.98]; $P = 0.028$ compared with 25.2 months of 716 placebo-treated patients [120]. Upon arrival of tumor cells in bone, osteoblasts produce PGE₂, which binds and stimulates their own receptor for PGE₂, EP₄, resulting in the induced expression of RANKL. EP₄ antagonist, AE3-208, could inhibit osteolytic metastasis by B16 melanoma cells after intra-cardiac injection [121, 122]. The same reagent also was potent to suppress lung metastasis by intravenously injected LLC and liver metastasis by intrasplenically injected MC26 colon cancer cells. Another group showed that the antimetastatic effect by EP₄ antagonists was abrogated in the background of NK-cell depletion or IFN γ -KO [63].

LPS-stimulated macrophages produce PGE₂ via induction of COX-2. PGE₂ can activate NF κ B in several systems through its receptors, such as EP₂ and EP₄, resulting in the establishment of the COX-2-PGE₂-EP-NF κ B-COX-2 loop. EP₄ activation by PGE₂ elicits both the cAMP effector pathway and the GRK-Src-PI3K- β -catenin pathway [123]. Nicotine promoted gastric tumor growth by activation of NF κ B inducing expression of miR16 and miR21 in a manner dependent on the simultaneously induced COX-2 and subsequent generation of PGE₂ acting on EP₂ or EP₄ [124]. Anti-EP₂ or -EP₄ siRNAs and their inhibitors suppressed NF κ B activation by nicotine. In endothelial cells of cerebral aneurysm, EP₂-mediated NF κ B activation induced CCL2 that participates in the formation of aneurysm [125]. NF κ B inhibition by decoy oligodeoxynucleotide impaired COX-2 expression. Thus, PGE₂-EP₂ and NF κ B is interdependent causing a vicious cycle. Furthermore, in an artificial system of HEK cells transfected with EP₄, PGE₂ stimulated the IL-8 promoter via NF κ B activation in a Src-dependent manner [126]. Combined inhibitory effects on NF κ B and EP₄ have been reported in Nexrutine, a bark extract from *Phellodendron amurense* with an anti-inflammatory activity in Chinese medicine, when applied to pancreatic cancer cell lines [127].

Fatty Acids

There are only two fatty acids that humans cannot synthesize: α -linoleic acid (ALA) and linoleic acid (LA). Depending on the position of the first double bond relative to the end, which is called omega position as opposed to the other terminal carboxyl group end, ALA is expressed as <18:3 omega-3> indicating that it has 18 carbon chain with 3 double bonds with the first one in the third position from the omega. LA is <18:2 omega-6>. From LA and ALA, longer fatty acids are generated, such as eicosapentaenoic acid (EPA) <20:5 omega-3,> docosahexaenoic acid (DHA) <22:6 omega-3,> and arachidonic acid (AA) <20:4 omega-6.> Humans can either synthesize EPA or just take EPA already synthesized by animal as food, such as cold

(continued)

water fish, whose high diet by the Greenland Eskimos is well-known for their low rate of ischemic heart disease.

Pro-resolving fatty acids are generated from those including LXA4 from AA, RvE1 from EPA, and RvD1, RvD2, and maresin1 from DHA. One unique feature of their biosynthesis is that the reactions take place transcellularly, i.e., during cell-cell interactions. For example, LTA4 that is synthesized in leukocytes serves as an intermediate for 12-LOX-mediated LX generation in platelets. Another interesting event is that aspirin-acetylated COX-2 loses its cyclooxygenase activity but alters its AA recognition site allowing conversion of AA to 15R-HETE in endothelial cells. Both EPA and DHA also are substrates for the acetylated COX-2. RvE and RvD were shown to be synthesized in hypoxic endothelial cells when exposed EPA or DHA in the presence of aspirin. DHA is converted to RvD1 and RvD2 in the absence of aspirin.

Resolvin E (RvE) and RvD

Using in vitro experiments, the Charles N. Serhan group showed that [3H]-RvE1 binds to recombinant BLT1, a LTB4 receptor, with KD 45 nM, and to membrane fractions of human polymorphonuclear leukocytes (PMN) with a Kd 48.3 nM, which is displaced by LTB4 with a Ki 0.08 nM but not chemerin peptide capable of binding ChemR23 [128]. Both RvE1 and LTB4 can transmit signaling, such as inhibition of adenylate cyclase activities as monitored by cAMP accumulation with EC50 being 3.2 nM and 0.015 nM, respectively, in forskolin-treated HEK cells expressing BLT1 [128]. In an in vivo zymosan-induced murine model of peritonitis in BLT1-KO mice, in which activated resident macrophages secreted LTB4 resulting in PMN recruitment in the cavity, the PMN influx was blocked by low doses of RvE1 in a manner dependent on BLT1. Those results indicated that RvE1 is a partial agonist for BLT1 (see Chap. 6 for pharmacological ideas). High doses of RvE1 suppressed PMN infiltration via ChemR23. ADP-stimulated platelet activation via P2Y12 was reduced by RvE1 binding to ChemR23 on the same platelets as judged by the surface P-selectin mobilization [129]. In the same peritonitis model with FPR2-KO mice, intraperitoneal accumulation of PMN was inhibited by RvD1 injection in an Fpr2-dependent fashion [130]. Low and high concentrations of RvD1 signal through GPR32 and FPR2, respectively.

I stated previously the roles of CNS in inflammatory aspect (see Chap. 12) in tumor progression. Cervical vagotomy reduced the intraperitoneal zymosan-induced pro-resolving responses, including acetylcholine, RvD1, and netrin-1, which was originally identified as an neuronal guidance molecule [131]. Proinflammatory responses balanced over the pro-resolving responses as represented by elevated LTB4, TNF α , IL-6, KC, and HMGB1, all of which were decreased by RvD1 administration.

Furthermore, they demonstrated that intrathecal (i.e., through spinal) administration of RvE1 inhibited neurogenic inflammation-induced pain provoked by intrathecal injection of capsaicin by reducing paw edema, PMN infiltration, and TRPV1-induced postsynaptic current [132].

Moreover, the same group by using a murine sepsis model by cecal ligation and puncture showed that intravenous injection of RvD2 prevented sepsis-related lethality not only by enhancing clearance of the bacteria in the peritoneal cavity but also by dramatically reducing the plasma levels of cytokines including IL-1 β , IL-6, TNF α , and IL-17 and numbers of intraperitoneal PMN [133].

RvD2 binds its receptor GPR18 with K_d ~10 nM and restricts infiltration of neutrophils [134]. In an ischemia-reperfusion-initiated model of lung injury caused by infiltrating neutrophils in a sterile condition, exogenous administration of RvD2 protected the lungs against damages in the wild-type but not GPR18-KO.

Thus, resolvins actively participate in resolution in both microbial and sterile inflammation and controlled by innervation (see Fig. 1.1 in Chap. 1).

16.4.3.2 Steroid

In addition to NSAID, another most used drug to reduce inflammation is glucocorticoid. It can suppress expression of COX2, iNOS, and ICAM-1 that is induced by cytokines, such as TNF α , in a manner counteracting against AP-1 and NF κ B.

Glucocorticoid is known to inhibit NF κ B activity and to induce death of immune cells, which may explain its efficacy, at least transiently, for the treatment of multiple myeloma and SLE. However, activation of TLR7 and TLR9 conferred protection against glucocorticoid-induced cell death of purified human pDC in which glucocorticoid did not affect NF κ B activities as judged by DNA binding and p65 phosphorylation [135].

The best setting for steroid to exert its valuable effect against cancer would be multiple myeloma. Although its pathogenesis remains to be uncovered completely (see Chap. 9), the essential nature of the disease is B-cell growth by differentiating and interacting with stromal cells in bone marrow. High-dose dexamethasone has been commonly used for treatment before the advent of a more effective drug, i.e., proteasome inhibitor bortezomib [136]. For 336 relapsed cases, the median time to progression was 3.49 months and 1-year survival rate was 66% under the dexamethasone treatment.

Given that genetically dominant clones in the metastatic site are present in the primary tumor (see Chap. 11 for details) as evidenced by clinical samples from pancreatic and renal cell carcinomas, metastatic dissemination could take place any time during tumor development, but the important point is whether or not the disseminated tumor can regrow in that particular distant organs as in the primary site. Only the population of cells that are allowed to regrow appears to manifest themselves as in those genetic analyses.

The Pdx1-Cre-based pancreas-specific expression of mutated K-Ras and p53 in combination with a Rosa-YFP allele integration to track down the cells gave rise to

pancreatic intra-epithelial neoplasia (PanIN) (see PIN in prostate cancer model in Chap. 11) [137]. Interestingly EMT was observed in these histologically defined precancerous PanIN in roughly 5% as judged by expression of EMT markers, such as Zeb1, Snail1, and Slug, i.e., at least one YFP⁺Zeb1⁺ cell found in 5% in PanIN with infiltration of inflammatory cells as well as YFP⁺ circulating pancreatic cells (CPC). Those CPC, which showed CD24⁺CD44⁺ stem cell features, were observed in both liver and lungs where they were located close to blood vessels. In this system, acute pancreatitis induced by either cerulean or pancreatic duct ligation enhanced PanIN formation with increased CPC numbers. Dexamethasone treatment dramatically reduced the inflammation, which was accompanied by elimination of PanIN and a significant decrease in CPC. Therefore, steroid appears to be effective to inhibit inflammation-associated EMT and intravasation.

16.4.3.3 The Issue of TNF α

Basic research results with mouse models indicate metastatogenic functions of TNF α . Lung metastasis after intravenous injection of LLC was dramatically abrogated in TNF α -KO mice but not IL-6-KO mice [62]. Conversely, we have shown that pretreatment with TNF α enhanced lung metastasis after intravenous injection of LLC. In SCID mice orthotopically transplanted with human pancreatic adenocarcinoma PancTu1 cells, infliximab reduced liver metastasis by 50% without and 69% with the primary tumor resection [138]. Conversely, treatment with TNF α in the same model enhanced liver metastasis [139]. What makes the story complicated is that TNF α also is secreted by tumor cells and TNF α receptor is expressed in a variety of types of cells, including tumor cells, fibroblasts, endothelial cells, and lymphocytes.

Like VEGF, TNF α appears play a defensive homeostatic role in the local microenvironment. In syngeneic orthotopic pancreatic cancer model with Panc02 cells, TNFR1(p55)-KO mice failed to reject the tumor growth. Conversely, however, systemic TNF α administration accelerated primary tumor growth with Treg infiltration but not liver metastasis [139]. Primary tumor growth of B16 and LLC cells in xenograft was reduced in TNFR2(p75)-KO mice suggesting an antagonizing role of the two TNFRs in tumor biology. Adenovirus-mediated TNF α expression in 187 locally advanced pancreatic cancer patients under the control of the Erg-1 promoter that is activated upon irradiation revealed neither tumor reduction nor progression [140]. Clinical research of 404 Crohn patients under infliximab revealed that TNF α blockade failed to show more tumor development than the noninfliximab group [141].

Taken together, anti-TNF α therapy could be effective in metastatic progression of tumor [142]. However, currently there is no such trial to prove although recombinant TNF α is approved for clinical use for sarcoma.

16.4.3.4 CXCL12

As I state in Chap. 11, CXCL12 is expressed in CAF and its receptor CXCR4 in tumor cells in a human breast cancer xenograft model. Anti-CXCR4 siRNA treatment resulted in tumor growth suppression. The premetastatic microenvironment with VEGFR1⁺ cells and fibroblasts (see Chap. 13) also is enriched in CXCL12 whose gradient attracts the CXCR4-expressing tumor cells in lung metastasis. The strategy to suppress the CXCL12-CXCR4 axis appears to be effective against metastasis.

Waldenstrom macroglobulinemia (WM), which manifests a B-cell lymphoproliferative disorder with monoclonal IgM elevation, was reported to have roughly 30% C1013G mutation in CXCR4 in 131 patients [143]. In a SCID/Bg murine model with WM cells expressing CXCR4, they disseminated to bone marrow, liver, and kidneys. When C1013G CXCR4 was introduced, the WM cells extensively spread in all the tissues, including lung and lymph nodes except for brain, which could be inhibited by anti-CXCR4 monoclonal antibody.

Because CXCL12 participates in HSC retention in bone marrow, the first-generation CXCR4 inhibitor plerixafor (AMD3100) was approved by the Food and Drug Administration in the United States for mobilization of HSC (see Chap. 11) in combination with G-CSF in blood diseases, such as malignant lymphoma. Although high expression of CXCR4 has been reported in a variety of metastatic human cancers, including breast, prostate, and ovarian cancers, other new CXCR4 inhibitors in clinical trial, such as MSX-122 and CTCE-9908, have so far as of 2014 no conclusion [144].

In addition to CXCL12, CXCR4 interacts with other molecules. In addition to CCR5, CXCR4 is utilized as a coreceptor with CD4 by HIV-1 to make an entry to the infected cells, which could be blocked by CXCR4 inhibitors under clinical trials. Unlike HIV-1 of exogenous origin, CXCL14 (see Table 2.2 in Chap. 2) binds CXCR4-expressing THP-1 cells with K_d 14.7 nM as conventionally evaluated by ¹²⁵I-labelled CXCL14, whose chemotaxis was suppressed by CXCL14 with IC₅₀ of 7.2 nM [145]. Cell migration of HUVEC in vitro that were stimulated by VEGF, bFGF, or IL-8 was inhibited by CXCL14. The similar inhibitory activities were observed in rat corneal in vivo neovascularization assay. Given that CXCL12 also stimulates endothelial cells in those two assays, CXCL14 may serve as an endogenous inhibitor of CXCL12-dependent cell mobilization and angiogenesis. Tumor growth of B16 or LLC was reduced with less tumor angiogenesis when implanted in transgenic mice of CXCL14 whose plasma level of CXCL14 was tenfold higher than the wild-type. C-terminal peptides capable of similar inhibition are under development.

16.4.3.5 CCL3 causes the Abscopal Effect

The abscopal effect is simultaneous regression of non-irradiated metastatic tumors that are located distantly from the irradiated tumor and has been observed infrequently in patients with lymphoma, esophageal carcinoma with lung metastasis, and melanoma [146]. The abscopal effect also was observed in mice transplanted with various tumors, including LLC under administration of a CCL3 variant called EC1301 [147]. The assumed molecular basis is recruitment of leukocytes expressing CCR1 and/or CCR5 and antibody-mediated depletion of lymphocytes or NK1.1 abrogated the effect.

16.4.3.6 SAA

A plasma cytokine profile analysis of 40 melanoma patients versus 30 normal individuals revealed up-regulation of GM-CSF, IL-8, IL-10, IL-13, and most prominently SAA1 [148]. SAA1 stimulates its receptor FPR2 in neutrophils to secrete IL-10. In a homeostatic feedback control of LPS as a trigger, for example, the secreted IL-10 plays an anti-inflammatory effect. As a double feedback, SAA1 also induced invariant NKT cell-mediated suppression of IL-10 but instead promotion of IL-12 secretion [148]. However, this oscillation caused by both positive and negative effects on inflammation is destroyed by constitutive expression of SAA1 from melanoma cells, resulting in promotion of immunosuppressive IL-10-secreting neutrophils. The authors found that a CD40-blocking antibody could abrogate the iNKT cell-mediated negative effect on the neutrophils.

16.4.3.7 S100A8 and S100A9

Overview

S100A8 is involved in three big problems in cancer treatment: metastasis (see Chap. 15), resistance against cytotoxic drugs (see below), and immune-suppression (see below). Good suggestions should be obtained from experimental results of endotoxemia, because S100A8 binds the same receptor TLR4 as LPS (see Fig. 15.3). Anti-lipid A antibody raised during sepsis is poor in its ability to defend against invasion by LPS-producing bacteria [149]. A TLR4 antagonist Eritoran failed to prolong the survival of septic patients [150].

Small Molecules

Here I introduce the studies by Tomas Leanderson group, which have identified inhibitory molecules against S100A9 that directly inhibit S100A9 binding to its

receptors, such as RAGE and TLR4. Quinoline-3-carboxamide derivatives inhibit binding of human S100A9 to human RAGE or TLR4 with IC₅₀ ranging from 60 to 400 μ M. One of the orally available ones, called tasquinimod (ABR-215050), increased PFS of castration-resistant prostate cancer patients in phase II clinical trial from 3.3 to 7.6 months against placebo [151]. It is assumed that tasquinimod modulates the expression of TSP-1 and down-regulates HIF-1 α , thereby impairing tumor angiogenesis. In an orthotopic SCID xenograft model of prostate cancer with androgen-independent LNCaP-19 cells intraprostatically implanted, tasquinimod dramatically inhibited lung and lymph node metastasis, which was accompanied by an increase of TSP-1 in the serum as well as in the primary tumor and decreases of HIF-1 α target genes, including VEGF, CXCR4, and lysyl oxidase (LOX). Importantly, when tasquinimod was applied 1 week after tumor cell implantation, frequency and nodule number in the lung metastasis decreased from 92% and 27 % to 0% and 0%, respectively, and the size of the primary tumor also was reduced by roughly 40%.

The same group additionally discovered a novel compound in salicylic amide derivatives called oxyclozanide, which inhibited the binding of S100A9-RAGE with IC₅₀ 1.2 μ M, S100A9-the TLR4-MD-2 complex with 1.6 μ M, S100A4-RAGE with 23.4 μ M, and S100A4-the TLR4-MD-2 with 25.7 μ M [152]. CD11b⁺Ly6G⁺ cells express high levels of S100A8 and S100A9, but they lack S100A4 expression. An opposite pattern of expression is observed in CD11b⁺ Ly6C⁺ cells, suggesting that not only tumor cell-derived S100A4 (see Chap. 15) but also MDSC-derived S100A4 could affect the tumor microenvironment as evidenced by the possible formation of heterodimer of S100A4-S100A9 when cotransfected in 293 cells [151].

Peptibodies

Peptibodies were obtained by competitive peptide phage library screening to isolate peptides that could bind MDSC. They bound both S100A8 and S100A9 on the surface of MDSC and depleted MDSC, both granulocytic and monocytic, in the syngraft, blood, or spleen of three different tumor models, including EL4 thymoma when administered intravenously [153]. They reduced the EL4 tumor size by more than 50% at 2 weeks after challenge. Their depletion effect on MDSC was partial in S100A9-KO mice, which can be understood considering that S100A8 expression is still observed, although S100A9 stabilizes S100A8 at protein levels. They failed to deplete DC or Gr1⁺CD11b⁺ cells in the bone marrow in tumor-bearing mice. I am really curious to see if they also can prolong the premetastatic period of lung metastasis.

TLR4 Antagonists

Many cancers, whether human or murine, express TLR4 [154]. For example, one study of 108 colorectal cancer patients showed that high expression of TLR4 and MyD88 is associated with liver metastasis [155].

TLR4-KO mice were refractory to lethal influenza infection (see Chap. 2) that causes lung injury in the lower respiratory tract through generation and release of oxidized phospholipid-like OxPAPC (see Chaps. 6 and 7) and presumably HMGB1 (see Chap. 7) and S100A8 from infiltrating leukocytes and necrotic cells. Eritoran also called E5564 protected those mice against lethality in a manner dependent on TLR4 and CD14 [156]. It was assumed that LPB-dependent binding of eritoran with CD14 is followed by binding of eritoran with MD-2. This was accompanied by reduced expression of cytokines, including TNF α , IL-1 β , CXCL1, and IL-6 in the lungs. If you take two of the cytokines, TNF α and IL-1 β , it was shown that lung injury caused by the highly virulent avian influenza H5N1 was reduced with significantly decreased levels of CCL2 and KC in the background of knockout of receptors for those cytokines, i.e., TNF-R1-KO, TNF-R2-KO, and IL-1RI-KO [157]. Collectively, influenza causes lung injury with a paracrine cytokine storm in which TLR4 and its undetermined endogenous ligands make a major contribution but are sensitive to eritoran.

However, the TLR4-influenza story is not so simple. LPS pretreatment could protect mice against H5N1 [158].

Both S100A8 and SAA3, endogenous TLR4 ligands, also are expressed in the primary tumor, premetastatic lungs and postmetastatic lungs. As expected, inhibition of TLR4 signaling by Eritoran resulted in decreased numbers of BMDC in premetastatic lungs [159]. Unexpected findings included reduced sizes of the primary LLC tumors in which BMDC numbers also were decreased. Suppressor functions of BMDC (BMSC), such as arginase-1 expression were impaired in both myeloid cells from the primary tumor and the pre-metastatic lungs of tumor-bearing mice.

The results raise a number of important issues. First, eritoran may inhibit reciprocal activation between HIF-1 and NF κ B. NF κ B is supposed to be activated persistently and at least locally by S100A8-TLR4 signaling in both primary tumors and premetastatic lungs. Inflammation and hypoxia may be interdependent, because NF κ B activates HIF-1 and hypoxia activates NF κ B [160, 161]. As described before, hypoxia exists in tumor microenvironment and bone marrow. Then does hypoxia exist in premetastatic lungs? Dissociation among hypoxia, perfusion, and HIF-1 α up-regulation as judged by pimonidazole, Hoechst33342, and anti-HIF-1 α immunostaining, respectively, was reported in the orthotopic 4T1ODD-luc tumor model treated by doxorubicin [162]. In an acute lung injury model by mechanical stretch, stretch inhibited succinate dehydrogenase (SDH) (see Chap. 10) in normoxic conditions. The resulting accumulation of succinate and succinate-elicited PDH inhibition was responsible for HIF-1 stabilization [163]. Nonhypoxic conditions, such as NO generation by VEGF-activated eNOS, also can lead to PHD inhibition-mediated activation of HIF-1 α at least transiently [164]. Those examples

suggest hypoxia-independent activation of HIF-1 α and subsequent induction of HIF-1 α target genes, such as angiogenic VEGF and LOX. Conversely, conditional knockout of HIF-1 α in macrophages impaired their functions and myeloid cell infiltration in the inflammatory tissues [165]. This implicates that the inflammation not only in primary tumor but also in premetastatic lungs with activation of recruited myeloid cells may involve hypoxia-mimicking conditions.

Second, given the analogy between LPS-driven mobilization of HSC and S100A8-stimulated recruitment myeloid and metastatic tumor cells in the lungs in a manner partly dependent on TLR4 expression in those cells (see Chap. 15), eritoran may inhibit mobilization of not only non-CSC but also CSC that are known to efficiently cause lung metastasis in some experiments (see above). This idea points out that eritoran seems to be an inhibitor against both soil (premetastatic microenvironment) and seed (tumor cells).

In the HGF-CDK4 (R24C) murine model of melanoma development and progression (see Chap. 12), HMGB1 (see Chap. 7) released from the damaged epidermal keratinocytes by UVB treatment activated TLR4, which, by an as yet unknown mechanism, promoted angiotropism (see Chap. 11) of melanoma cells followed by lung metastasis [166]. The extracellular HMGB1 was reflected in its increased serum levels. Prominent infiltration of CD11b⁺Ly6C⁺Ly6G⁺ neutrophils in the UVB-exposed dermal melanoma, which could be substantially reduced not only by the genetic background of TLR4 or MyD88, but not of TRIF, but also pharmacological inhibitors of HMGB1, including recombinant HMGB1 Box A (see Chap. 6) or glycyrrhizin or that of TLR4 signaling, such as CLI-095 (also called Tak-242 or resatorvid). The angiotropism and spontaneous lung metastasis in the skin transplant of HcMel12 melanoma cells derived from the DMBA-treated HGF-CDK4 (R24C) mice increased in a UV-dependent manner, which also was abrogated by TLR4-KO, MyD88-KO, above-mentioned pharmacological inhibitors and importantly by antibody-mediated depletion of Ly6G⁺ neutrophils. Those results show the same logics to the premetastatic lung microenvironment that is supported by endogenous ligand-mediated TLR4 activation and myeloid cell infiltration.

Glycyrrhizin is commonly used for chronic viral hepatitis in clinical settings by its glucocorticoid activity. In addition, it directly binds HMGB1 as evidenced by NMR studies with K_d 150 μ M and inhibits HMGB1-stimulated migration of 3T3 cells with IC₅₀ around 49 μ M [167].

In animal models of both ischemic and hemorrhagic brain, glycyrrhizin reduced the brain damage [168]. In the latter case, thrombin-induced cortical injury was decreased in a concentration-dependent manner.

In ischemia-reperfusion model of brain MCA in mice, anti-HMGB1 antibody reduced vascular permeability as monitored by Evan blue 3 h after reperfusion, number of activated microglia as judged by *Griffonia simplicifolia* (IB4)-lectin but not that of neutrophils [169]. Increased vascular permeability augments hypoxia in the brain resulting in further release of HMGB1. The same group performed the Evans Blue experiments under the genetic background of TLR4-KO, TLR2-KO, or RAGE-KO and found that the HMGB1 inhibition is effective only in the RAGE-KO [170]. However, it can easily be assumed that it depends on the organ.

16.4.3.8 How to Think of HMGB1 Binding to CXCL12

Considering that HMGB1 binds CXCL12, HMGB1 blockade shows a significant effect on neutrophil infiltration [171]. Cell migration in vitro of NIH3T3 cells by HMGB1 was inhibited by roughly 50% by anti-CXCR4 blocking antibody or CXCR4 antagonist AMD3100. HMGB1 induced CXCL12 secretion in a RAGE-dependent manner, which was followed by physical binding between HMGB1 and CXCL12. The HMGB1-CXCL12 complex now stimulated more recruitment of neutrophils in subcutaneously established air pouches than CXCL12 alone in a manner dependent on CXCR4 but not RAGE, TLR4, or MyD88 as judged by results of cell migration assays with bone marrow cells derived from knockout mice for each of them.

16.4.3.9 S100A8/A9-AA Binds CD36

CD36 is a PRR that recognizes poly-anionic ligands, such as oxidized LDL (oxLDL) and fibrillar amyloid β by cooperating with TLR4/6 [172, 173]. Soluble oxLDL outside of cells is crystalized intracellularly resulting in NLRP3 inflammasome activation. Macrophages derived from individual KO mice of CD36, TLR4, or TLR6 failed to produce IL-1 β by stimulation with oxLDL. S100A8-A9 bound to arachidonic acid (AA) was reported to be rapidly taken up by HUVEC in a manner mediated by CD36 and fatty acid transporter [174]. In addition, S100A8/S100A9 prepared with endotoxin less than 10–4 EU/ml induced secretion of IL-6, IL-8, and IL-1 β in peripheral blood mononuclear cells by activating NF κ B and NLRP3, which was dependent on the generation of ROS [175].

In a murine model of Alzheimer disease, a synthetic inhibitor called FPS-ZM1 capable of blocking interaction between HMGB1 or S100B and a soluble form of RAGE with K_i of 148 nM or 230 nM, respectively, suppressed microglia activation by amyloid β peptide binding RAGE and normalized cognitive performance [176]. Given RAGE also binds S100A8 the RAGE inhibitor might be useful for blocking S100A8-mediated tumor biology.

16.4.3.10 S100A9 Binds CD147 (EMMPRIN)

Whereas S100A9 stimulates CD147 to induce secretion of cytokines, such as CXCL1 and TNF α , as I stated in Chap. 15, CD147 can be activated by an enzymatic ligand, the extracellular cyclophilin A [177]. Cyclophilin A is a well-known signaling molecule for the immunosuppressive drug cyclosporine A responsible for protein folding and cytokine production. However, it also has been known that a soluble form of cyclophilin A is present in synovial fluid of rheumatoid arthritis patients and is released from activated platelets. This extracellular cyclophilin A

has a peptidyl-prolyl cis-trans isomerizing activity for the extracellular domain of CD147, resulting in intracellular signaling, for example cell growth in tumor cells by Erk activation. The principle is similar to PAR-1 activation by thrombin (see Fig. 3.5). An anticyclophilin blocking antibody or nonimmunosuppressive cyclosporine A analogue NIM811 reduced the neutrophil infiltration by roughly 50% in the LPS-induced acute lung injury [178].

16.4.3.11 CCL2-CCR2 Signaling Block

The Mathias Heikenwalder group showed up-regulation of CCL2 in colon cancer, which stimulated CCR2 in endothelial cells to enhance vascular permeability, extravasation and metastasis [179]. The CCL2-CCR2 signaling was mediated by JAK2-Stat5 and p38 [180]. In their nice review of the CCL2-CCR2 [179], by pointing out antitumor effects of host-derived CCL2, they referred to a possible existence of switching mechanisms by which to convert anti- to prometastatic activity in a manner dependent on cellular context, tumor types, i.e., some tumor cells can metastasize independent of CCL2, and cancer stage, and to clinical trials of CCL2-CCR2 blockade against metastatic tumors.

CCL2 overexpression of breast cancer correlated well with metastatic progression and poor prognosis. Experiments to support the roles played by the CCL2-CCR2 signaling in homeostatic inflammation and metastasis are described in Chaps. 14 and 15. In the orthotopic mouse model of human breast cancer MDA-MB-231 cell, anti-human CCL2 and anti-mouse CCL2 antibody individually suppressed spontaneous lung metastasis, indicating that CCL2 derived from not only tumor cells but also stromal cells promoted lung metastasis.

16.4.3.12 CXCL1-S100A8 Loop

In 2012, the Joan Massague group showed an intriguing vicious cycle of CXCL1-S100A8 that gives TNF α -mediated resistance to chemotherapy [181]. In either an orthotopic allograft model of PyMT mammary cancer cells or orthotopic xenograft NOD-SCID model of LM2-4175 lung metastatic cells derived from MDA-MB-231 human breast cancer cells, they found that tumor-derived CXCL1 and CXCL2 stimulated recruitment of MDSC expressing their common receptor CXCR2 from bone marrow in both the primary tumor and the lungs. The MDSC produced S100A8 and S100A9. Importantly, the CXCL1/2 knockdown tumors failed to recruit MDSC in the primary site and the lungs without affecting the cell numbers of macrophages and CAF, which was rescued by forced expression of S100A8 and S100A9 in those cells. Although it was a postmetastatic condition, they added that higher the S100A8 and S100A9 levels in the metastatic nodules in the lungs of breast cancer patients, the shorter the overall survival compared with the lower levels of S100A8 and S100A9. S100A8 and S100A9 stimulate both the TLR4/MD-

2 complex and RAGE both of which are expressed in breast cancer cells leading to NF κ B activation, which targets the CXCL1/2 genes.

Chemotherapy by doxorubicin, cyclophosphamide, and paclitaxel up-regulated production of TNF α in both clinical samples and experimental conditions of breast cancer, further enhancing the CXCL1 secretion from the tumor cells. The aggravating loop could be inhibited in an experimental condition with the LM2 cells in which a CXCR2 inhibitor was applied after lung metastasis was detected by bioluminescence, giving more than 90% reduction relative to chemotherapy alone.

Double-Edged Swords

I stated above that TLR4 antagonists are useful for influenza lethality and melanoma progression both of which are mediated by TLR4. However, at the same time I referred to protection against H5N1 by pretreatment by LPS, an authentic TLR4 agonist. Clinical trials are undertaken for TLR agonists that may augment anticancer immune responses including BCG (bacillus Calmette-Guerin) from *Mycobacterium bovis* and monophosphoryl lipid A (MPL) from *Salmonella minnesota* (see adjuvant in Chap. 6) [182]. Both of them are well-known TLR4 agonists. Depending on the phase and biological context, for example, on which immune response dominates in the situation, innate or acquired, or on the presence of partial agonists (see Chap. 6), TLR4 can serve as a double-edged sword.

In addition, even in acquired immunity, we need to think of an idea of blocking antibody. Raised antibodies against LPS, for example, do not necessarily neutralize the harmful activities of the LPS-bearing bacteria. Careful analysis of sputum derived from bronchiectasis patients with chronic *Pseudomonas aeruginosa* infection revealed the presence of IgG2-type blocking antibodies against the O-antigen side chains, but not lipid A of LPS, which in excess binds and shields the bacteria against complement-dependent killing. Elimination of the blocking antibodies could restore the killing ability back to the patient serum [183].

16.4.3.13 S1P

I reviewed the basic biochemistry and mechanisms of release of S1P from platelets in Chap. 3. Here I describe its complicated linkage to tumor progression and potential usefulness to strategize against it [184].

When SK1 was overexpressed in NIH3T3 cells by sixfold to achieve increased intracellular, but not extracellular, S1P levels in a manner sensitive to inhibition by an SK blocker N,N-dimethylsphingosine, the cells manifested anchorage-independent growth in soft agar and tumor formation in NOD/SCID mice [185]. Erk is known to phosphorylate SK1 to induce its translocation to the plasma membrane to acquire high enzymatic activity [186]. Transformation of NIH3T3

cells by V12-H-Ras was inhibited by co-expression of a dominant-negative SK1G82D that lack the lipid kinase activity or SK1S225A that is unable to be phosphorylated for the translocation by roughly 50%. A variety of human cancers have elevated expression of SK1 including stage IIIA breast cancer patients. A specific SK1 inhibitor, BML-258, reduced lung and lymph node metastasis by suppressing both angiogenesis and lymphangiogenesis in a syngeneic murine mammary cancer model with 4 T1-luc2 cells [187].

It is known that the S1P-S1P1 pathway stimulates migration in monocytes and tumor cells. Here I introduce interesting experimental results by the Hua Yu group. They found that S1P1 and phosphorylated Stat3 (pStat3) were coexpressed in a variety of human cancers, including breast cancer, prostate cancer, renal cell carcinoma, and lymphoma [188]. Microarray analysis between tumor-derived myeloid cells and normal spleen myeloid cells revealed elevated expression S1P1 in the former. Conversely, the B16F10 tumor as a whole exhibited reduced growth and expression of S1P1 when grown in mice under the background of an inducible myeloid cell-specific ablation of Stat3 by the Mx1-Cre-loxP system. The ablation is achieved by injecting poly (I:C) capable of activating TLR3, but they showed that the injection just transiently induced activation of DC as monitored by expression of TNF α , IL-12, and IFN α . Blocking Stat3 by an inhibitor CPA-7 dramatically suppressed lung metastasis by intravenous injection of B16 melanoma cells. They showed that S1P activates Stat3 via S1P1 recruited with Jak2, which lasted longer than 18 h in splenocytes while IL-6-activated Stat3 subsided down within 1 h under the same experimental condition.

Intriguingly, the persistent S1P1-Stat3 signaling in tumor cells can induce the same signaling in CD11b⁺ myeloid cells that were cocultured with the tumor cells [189]. A premetastatic lung microenvironment could be established by intravenously injecting tumor-conditioned medium from S1P1-overexpressing B16 melanoma cells for 5 days [190]. The procedure induced up-regulation of not only S1P1, pStat3, and LOX in the recruited CD11b⁺ myeloid cells but also of fibronectin in the lungs. The subsequent injection of tumor cells through the tail vein promoted lung metastasis.

Myeloid cell-specific ablation of S1P1 in Mx1-Cre-S1P1lox/lox mice also reduced pStat, myeloid cell recruitment and metastasis in the lungs. They also showed that a Jak2 inhibitor AZD1480 (see *Tyrosine Kinase Inhibitor* section below) suppressed CD11b⁺ cell recruitment in premetastatic conditions and spontaneous lung metastasis in orthotopic 4 T1 mammary tumor model. Moreover, they showed increased expression of S1P1 and pStat3 in lymph nodes of advanced prostate cancer and melanoma patients. The mechanism may be increased serum levels of S1P in hosts that have S1P1-overexpressing tumors at the beginning, which induces up-regulation of both S1P1 and IL-6 in myeloid cells in the premetastatic lungs. After the tumor cell arrival the S1P-Stat3 signaling is supposed to be amplified. It is noteworthy that pStat3 induces IL-6 and IL-10, and IL-10 promotes poly (I:C)-stimulated expression of S100A8 and that S100A8 was expressed in the influenza-infected murine lungs. It is likely that IL-10 maintains the expression levels of S100A8 [191].

16.4.3.14 PPAR γ Agonists

Synthetic ligands of PPAR γ , such as rosiglitazone and pioglitazone, are frequently used for the treatment of diabetic patients. The endogenous agonists include unsaturated fatty acids and 15-hydroxyeicosatetraenoic acid (HETE). The corepressor complex containing HDACs and NCOR suppresses transcription of inflammatory genes, such as CCL2 in noninflammatory conditions. LPS stimulation-induced TLR4 signaling promotes degradation of the corepressor complex by proteasome, which is concomitant with NF κ B activation. When PPAR γ is activated by binding to its ligands, SUMOylated PPAR γ binds and stabilizes the corepressor complex to be resistant to degradation [192]. While TLR4 signaling favors M1 macrophages, IL-4-Stat6 signaling up-regulates PPAR γ and its ligands to favor M2 [193].

When subcutaneously grown human hepatocellular carcinoma MHCC97L cells were implanted in the liver of nude mice, rosiglitazone reduced lung metastasis [194].

16.4.3.15 Tyrosine Kinase Inhibitor

Met Inhibitors

Instead of targeting tyrosine kinases that are expressed in endothelial cells, such as VEGFR2, or tumor cells by themselves to reduce their potency, I describe drugs to suppress cytokine release and vascular permeability both of which constitute the major events in inflammation.

Up-regulation of c-Met has been observed in resistance against not only antiangiogenesis therapy (see Chap. 12) but also anti-EGFR agents, such as gefitinib [195]. Its ligand HGF activates the Ras-MAPK, PI3K-Akt, NF κ B, and Stat3 pathways and cross-talks with Wnt signaling to exert a pleiotropic biological effects. Inhibitors of c-Met are currently under clinical trials and some of them have given promising results. For example, in 171 patients with castration-resistance prostate cancer, cabozantinib capable of blocking both c-Met and VEGFR2 had clinical benefit by reducing the soft tissue lesions in 72% of the evaluated patients [196].

Jak2

Although the discovery of an activating mutation of JAK2 (V617F) frequently in myeloproliferative disorders (MPD), including polycythemia vera, essential thrombocythemia, and myelofibrosis, appeared promising as in the case of BCR-ABL in CML, JAK2 inhibitors failed to reverse the diseases [197]. However, one of them called ruxolitinib showed valuable effects, such as spleen size

reduction in myelofibrosis (see Chap. 9 for discussion of stem cells in spleen), whether primary or secondary to MPD [198, 199].

BCR-ABL is known to form a complex with JAK2 and AHI-1 adaptor. Combination therapy of imatinib and a JAK2 inhibitor TG101209 induced destabilization of the complex, CML progenitor cell death in both in vitro and in vivo as monitored by LTC-IC assays, and improved survival of NSG leukemic mice more efficiently than imatinib alone [200].

JAK2 inhibitors could be useful in tumors in which JAK2 activated by cytokines promotes metastasis. High expression of IL-6 was observed in 78% of the metastatic tissues including regional lymph nodes in 87 prostate cancer patients [201]. IL-6 receptor also is expressed in prostate cancer cells and IL-6 stimulated cell migration of human prostate cancer DU145 cells. DU145 cells infected with IL-6 lentivirus not only increased adhesive ability to HUVEC in vitro but also enhanced lung metastasis by sevenfold when injected through the tail vein of nude mice, which was 70% diminished by either shRNA-mediated knockdown of Stat3 or a JAK2 inhibitor AZS1480.

Syk

Syk is activated via ITAM in FcγRs (see Chap. 2), a mediator in TNFα-induced production of cytokines and MMPs, and a proposed signaling TK in TLR4 activation. Fostamatinib is a prodrug, i.e., converted to active metabolites after oral administration to inhibit Syk and was shown to be effective in subclinical setting, such as collagen-induced arthritis. Treatment of 189 patients with MTX-resistant RA with fostamatinib revealed significant benefits as judged by the criteria of the American College of Rheumatology 20% improvement (ACR20) at 4 months [202]. Clinical features included rather rapid response at 1 week, and serum levels of IL-6 and MMP-3 were decreased at 4 months with neutropenia as one common side effect.

ABL

As already mentioned in details, imatinib has made an unprecedented contribution to CML therapy. The spectrum of inhibited TK includes ABL, c-Kit, PDGFR, and CSF1R with IC50 ranging from 0.1 to 1.5 μM, all of which readers can now understand contribute to tumor microenvironment. In a murine model of collagen-induced arthritis, imatinib reduced its incidence, paw swelling, erythema, thickness, and joint rigidity by suppressing c-Kit in mast cells releasing TNFα and IL-6, CSF1R in macrophage activation and PDGFRβ in fibroblast proliferation [203]. Needless to mention imatinib appears to play a major contribution in this setting.

Establishment of endothelial cell-specific conditional KO of ABL in Ablflox/flox; Arg+/-; Tie2-Cre+/- mice revealed that ABL kinase activity is required for

disruption of adherens junction-mediated endothelial barrier, i.e., enhanced vascular permeability by VEGF and thrombin [204]. Imatinib could impair the vascular permeability induced by thrombin and histamine. The updated summary of experience of CML treatment by the ABL inhibitors has revealed that attention needs to be paid on the adversary vascular events [205]. Molecular mechanisms involve activation of barrier-stabilizing Rac1, decreased activation of Rho, and reduced phosphorylation of MLC in endothelial cells treated by VEGF or histamine. Moreover, fibronectin (Fn)-induced phosphorylation of paxillin that resulted in promoting cell migration took place in a VEGFR2-independent but NP-1-dependent manner in human dermal microvascular endothelial cells [206]. Remember that NP-1 usually couples with VEGFR2 but they showed that ABL couples with NP-1. TGF β -stimulated cells secrete fibronectin in a form of soluble dimer, which is converted to insoluble fibrils by interaction with integrins increasing the stiffness of the ECM that supports the tumor growth, in which both ABL and NP-1 are essentially involved [207]. MEF from NP-1 flox/flox mice that were treated with adenovirus-Cre to knockout NP-1 were mixed with LLC before implantation to NP-1flox/flox-SMCre mice in which NP-1 is genetically depleted in myofibroblasts resulted in reduced tumor growth. Thus imatinib could affect Fn fibril assembly in tumor microenvironment. In addition to ECM as represented by Fn, PlGF stimulation of NP-1 in medulloblastoma is described in Chap. 12.

ABL and its closest homolog ARG also are activated in T cell signaling elicited by CXCR4 and CCR7, which induces ABL-mediated tyrosine phosphorylation of CrkL. The biological consequences include cell migration to lymph node and sites of inflammation [208].

16.4.3.16 mTOR Inhibitors

Originally, the target of a macrolide antibiotic rapamycin is mTOR, a master regulator of protein synthesis. It is known that rapamycin can extend life span in yeast, nematode and mice [209]. Depending on its partner, raptor or rictor, mTOR forms a complex mTORC1 or mTORC2, respectively. Generally mTOR is located downstream of PI3K-Akt and Ras-Raf-Erk signaling and its activity is increased in a variety of tumor tissues.

TNF α -induced VCAM-1 expression in HUVEC enhances adhesion to T cells through VLA-4 ($\alpha 4\beta 1$ integrin), which was blocked by mTORC2-mediated inhibition by rapamycin [210]. PMN cells can be recruited to endothelial cells and release TNF α in response to LTB $_4$, C5a, and CXCL1 (KC), but plasma protein leakage as judged by Evans blue was abolished in TNFR-KO mice, underscoring the importance of TNF α in the local inflammatory microenvironment [211].

mTOR is known to be activated in human advanced prostate cancers. Sequencing ribosome-protected mRNA fragments to determine on-going translation-specific mRNAs revealed 144 mRNA species that were targeted by an mTOR ATP site inhibitor PP242, among which included metastasis-promoting genes, such as YB1, vimentin, CD44, and MTA1 [212]. They newly developed a highly potent mTOR

inhibitor called INK128 with K_i 1.4 nM derived from PP242. In PTEN-KO mice model of prostate cancer progression, INK128 completely blocked local invasion as well as lymph node metastasis, which was accompanied by decreased expression of YB1, vimentin, CD44, and MTA1 at the protein level but not mRNA level.

As stated, cancer cells are addicted to glucose and glutamine (see Chap. 10). When cells transformed by activated Ras or Src were cultured in the deprived condition of amino acids but supplied with albumin, they engulfed albumin by macropinocytosis with subsequent proteolytic degradation generating amino acids including glutamine [213]. EIPA, 5-(N-ethyl-N-isopropyl) amiloride, could inhibit the process. Application of EIPA to Ras-transformed pancreatic tumor xenografts showed inhibition of tumor growth. Given that the utilization of extracellular protein as nutrients in cancer cells in amino acid-starved cells is suppressed by mTORC1, we need to be cautious in treating patients with mTOR inhibitors [214]. Not only extracellular proteins but also secretory exosomes that contain various molecules, including lipids, RNA, and proteins are up-taken in cells activated by Ras and EGFR [215]. Given the integrin-dependent directing property of pathologically produced exosomes from tumor cells in premetastatic microenvironment (see Chap. 13), exosome-mediated drug delivery system might be one of the future therapeutic applications [216].

16.4.3.17 Targeting microRNA

Initially discovered as a developmental regulator in *C. elegans*, accumulating evidence shows that targeting microRNA (miR) controls gene expression by locating both upstream and downstream of transcription factors [217]. Transcription by RNA pol II is followed by cytoplasmic processing by Dicer and unwinding to give rise to single-stranded mature miR [218]. The miR after being integrated to miR-protein complexes guides them to the target mRNAs for their destabilization. For the terms, antagomiR is antisense oligonucleotide, and oncomiR and metastamiR are miRs involved in cancer and metastasis, respectively [219]. Search for miRs essential for a certain pathological condition is therapeutically promising in terms of the fact that they are endogenous molecules as in the case of insulin in diabetes mellitus. For example, kinetic balance between calcium influx by SERCA and efflux via ryanodine receptor in sarcoplasmic reticulum regulates contractility in cardiomyocytes. It was found that miR-25 suppresses SERCA mRNA and its antagomiR against miR-25 inhibited heart failure [220].

Twist induced miR-10b resulting in up-regulation of metastasis-promoting RhoC and metastatic breast cancer samples from patients had elevated expression of miR-10b [221]. Another study showed that miR-335 was lost in relapsed breast cancers, which was closely associated with metastasis-free patient survival [222]. Expression of both metastasis-promoting tenascin C (see Table 7.1 in Chap. 7) and Sox4 was suppressed by miR-335 that is expressed in normal

human mammary organ. Located downstream of breast cancer metastasis suppressor 1 (BRMS1), miR-146 inhibited metastasis of MDA-MB-231 human breast cancer cells by decreasing NF κ B levels [223]. Moreover, miR-34a, which is a p53 target, was found to be down-regulated in CD44⁺ prostate cancer cells [224]. Lipid-mediated systemic delivery of miR-34a in an orthotopic model with LAPC9 prostate cancer cells resulted in abrogation of lung metastasis [224]. It turned out that miR-34a decreased CD44 expression. Conversely, an antagomiR against miR-34a (anti-sense inhibitor of miR-34a) in CD44⁺ cells promoted lung metastasis.

Furthermore, miR-34a was found to be a suppressor for osteoclastogenesis. In intra-cardiac injection of a human breast cancer MDA231-BoM-1833 cells in nude mice and of B16-F10 melanoma cells in allogeneic mice, bone metastasis was diminished in osteoclastic miR-34a transgenic strategy and could be reduced by miR-34a nanoparticle treatment in a manner directly dependent on its target TGF- β -induced factor 2 (Tgif2) [225]. As of 2014, MRX34 is the first cancer-targeted miR drug, a liposome-mediated miR-34 mimic, in clinical trial in advanced hepatocellular cancer patients [226].

16.4.3.18 CRP

As stated in Chap. 1, CRP represents one of the most reliable biomarkers in inflammatory disorders. It is known to activate complements thereby aggravating the tissue injury as in the case of myocardial infarction. In contrast to human, CRP elevations in rodents in response to inflammatory events are poor, which has been a reason why CRP research has been hampered. A specific small molecule inhibitor, 1,6-bis (phosphocholine)-hexane, was synthesized and bound human and rat CRP with K_d around 300 nM and 150 nM, respectively. The IC₅₀ for CRP binding to pneumococcal C-polysaccharide was 2 μ M [227].

Coronary artery ligation combined with subcutaneous injection of human CRP resulted in larger infarcts with increased mortality in rats than ligation alone, which could be rescued by prior administration of this reagent. Pure human CRP alone without LPS contamination is not proinflammatory, nor is its transgenic mice proatherogenic in the background of apolipoprotein-E-KO [227].

As previously mentioned, CRP binds both Fc γ RIA and Fc γ RIIC with ITAM (see Chap. 2). Myeloma cells express both of them but lack Fc γ RIIB with ITIM. Thus, CRP not only enhances proliferation of myeloma cells but also confer resistance on them to drug-induced apoptosis via NF κ B activation [228]. In a SCID mice model with subcutaneous implantation of human myeloma cell lines, such as ARP-1, administration of dexamethasone or melphalan reduced the tumor growth. However, injection of CRP alone failed to influence the tumor growth but protected the tumor against the pharmacological reagents. There seems to be a vicious cycle between hepatocyte-produced CRP and myeloma cell-secreted IL-6. Therefore, it is a reasonable strategy to target CRP in multiple myeloma.

16.4.3.19 Miscellaneous Targets

HDAC

Valproate, which has been used for epilepsy as an anticonvulsive drug that increases the inhibitory neurotransmitter GABA by blocking GABA transaminase, was shown to bind and inhibit HDAC [229]. Valproate inhibited primary tumor growth of MT-450 rat breast cancer cells that were subcutaneously implanted in rats and their lung metastasis.

Hypoxia repressed RECK expression in human breast cancer cell lines such as H-Ras MCF10A in an oxygen concentration-dependent manner, which was reversed by an HDAC inhibitor (iHDAC) trichostatin A (TSA) thereby suppressing the activity of both MMP2 and MMP9 [230]. An siRNA-mediated silencing of HDAC1 also counteracted against hypoxia-induced down-regulation of RECK and decreased cell migration. Given that hypoxia confers both metastatic potentials and chemo-resistance on tumor cells in general, iHDAC is expected to inhibit tumor metastasis. However, a set of structurally different iHDAC, including valproate and TSA, augmented expression levels of conventional PKC (α , β 1 and γ) and novel PKC θ and decreased those of antiproliferative PKC δ , which was accompanied by hyper-acetylation of histone H3 and H4. Injection via tail vein of labeled three different human hepatic and lung cancer cell lines, including RFP-labeled Hep3B, caused metastasis in both lungs and liver in 2 months, which was enhanced by iHDAC treatment [231]. The authors proposed that iHDAC monotherapy may not be useful. Given that HDAC abrogation not only causes tumor cell death passively releasing intracellular molecules but also actively induced acetylated HMGB1 release [232], simultaneous suppression of endogenous mediators may be necessary.

SNS Blocker

An involvement of SNS in primary tumor was described in Chap. 12. SNS also innervates bone marrow and lungs. Chemical sympathectomy by norepinephrine analog 6-hydroxydopamine (6-OHDA), which is taken up by sympathetic nerves and transported to the termini to be oxidized generating ROS, reduced mortality in a murine influenza model with diminished leukocyte recruitment in the lungs and totally suppressed the biphasic IL-1 β elevations at days 2 and 6 postinfection [233]. An α -blocker phentolamine also prolonged survival. Efficacy of Eritoran, a TLR4 antagonist, is described in a murine influenza model (see Chap. 7). IL-1 β secreted from stromal cells or tumor cells also promotes invasiveness as assessed by B16 melanoma cells implanted in IL-1 β -KO mice [234]. Collectively, 6-OHDA may reduce primary tumor growth and lung metastasis.

Midkine

Midkine (MK) levels in blood are increased in patients of breast cancer and rheumatoid arthritis (RA). Similar to S100A8, it is abundant in synovial fluid of RA patients. Infiltration of inflammatory leukocytes in models of ischemia/reperfusion in kidney and anti-collagen- and LPS-mediated arthritis was abrogated in midkine-KO mice, suggesting its strong involvement in inflammatory process [235]. Midkine binds heparin as well as a variety of cell-surface receptors, including LRP-1, integrin $\alpha 4\beta 1$, and syndecans, and therefore its functional modes are still elusive. However, an anti-midkine monoclonal antibody was capable of suppressing the lung metastasis of an osteosarcoma cell line 143B that was injected via tail vein or implanted as orthotopical xenografts in SCID mice [236].

Osteopontin

Dramatic increase in osteopontin (OPN) in adipose tissues is associated with insulin resistance and numbers of crown-like structure and infiltrating macrophages are blunted in OPN-KO mice [237]. OPN stimulates migration of tumor cells presumably via integrin $\alpha v\beta 3$ and CD44v6. In a K-RasG12Dp53fl/fl mice in which intranasal inhalation of adenovirus-CMV-Cre can activates the mutated K-Ras expression and ablation of p53 resulting in lung cancer. Those tumors are removed from the lungs and implanted subcutaneously to SCID/beige mice to induce lung metastasis. In this system, anti-OPN antibody has a modest effect on the metastasis but the size of the primary tumor was not changed [238].

16.5 Concluding Remarks

The clonal selection hypothesis of cancer suggests that resistance against antineoplastic drugs are acquired during the tumor progression and those clones can disseminate throughout the body. However, metastasis can happen at any stages of tumor progression from highly invasive to preneoplastic or even normal cells can move via circulation in a homeostatic manner. Therefore, metastasis is never the final phenotype of the tumor. Inflammation is present in tumor. The inflammation can spread to other parts of the body than the primary site and change (cultivate) the microenvironment (soil) in remote sites before arrival and regrowth of tumor cells (seed). Thus, the botany (primary tumor) can expand its territory (field) through seed allowing its progenies to grow at distant sites (metastatic tumor). The regrowth of metastasizing tumor cells start another inflammation there. Then why not treat cancer patients with anti-inflammatory drugs?

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