Results and Problems in Cell Differentiation 62

Malgorzata Kloc *Editor*

Macrophages

Origin, Functions and Biointervention

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Mouse macrophages in scanning electron microscopy (SEM). Bar is equal to 10 μm. Image: M. Kloc and Kenneth Dunner Jr (MDACC High Resolution Electron Microscopy Facility)

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Preface

The macrophages are the most versatile and still little understood cells of immune system. Phenotypically and functionally, the macrophages are exceptionally unique cells. They combine evolutionarily ancient, unicellular, amoeba-like attributes, such as ability to actively move and devour (phagocytize) prey, with evolutionarily modern abilities to function as immune sentinels, responders, and effectors within the multicellular/organismal environment. In their beneficial immune capacity, the macrophages safeguard tissue and organ homeostasis and play an active role in wound healing and innate and adaptive immunity. But they can also be detrimental by promoting inflammation, vascular and autoimmune diseases, and cancer metastasis. Not until recently it has been discovered that, at least in mammals, not only macrophage functions but also their origin is multifarious: some macrophages descend from the embryonic yolk sac and fetal liver and some from the adult bone marrow precursors and circulating monocytes. This volume describes the ontogeny of macrophages, their evolutionary origin, various aspects of macrophage properties and functions in health and disease, and potential use of macrophages as a target for clinical interventions.

The first section of the book concentrates on evolutionary aspects of macrophage origin and polarization in invertebrates and vertebrates, ontogeny and functions of tissue-resident macrophages, and the origin and function of Hofbauer cell-specialized fetal macrophages residing in the mammalian placenta and describes how phenotypical and functional fate of macrophages is determined and modulated by mesenchymal stem cells and cooperation with NK cells. The second section of the book focuses on macrophage immunobiology describing how different locales, mechanical forces, and inflammatory environments regulate expression of macrophage markers and functionally active molecules. The third section discusses the role of macrophages in vascular and respiratory diseases, wound healing, and regeneration. Finally, the fourth section outlines various potentially clinically applicable methods and biomaterials that can be used to manipulate macrophage functions and responses in wound healing, regeneration, and transplantation.

I dedicate this volume to my dear friend Dr. Rafik Mark Ghobrial who transplanted me from the germ cells and developmental biology métier to the realm of immunology.

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Abstract

Macrophages are evolutionarily conserved phagocytic immune cells. In recent years, macrophages have emerged as one of the most versatile cells of immune system, which, depending on the milieu and circumstances, participate in innate and adaptive immune responses, inflammation, regeneration, wound healing, development or inhibition of cancer, organ rejection, and interaction between a mother and a fetus.

This volume gives an overview of recent knowledge on macrophage functions in various vertebrate organs, systems, and diseases. In addition, the volume covers various aspects of macrophage development, formation, behavior, and response to biomaterials and other potentially clinically applicable methods of biointervention.

Contents

Part I
Evolution, Origin and Fate of Macrophages

Chapter 1
Evolutionary Aspects of Macrophages Polarization

Eva-Stina Edholm, Kun Hyoe Rhoo, and Jacques Robert

Abstract Macrophages constitute a heterogeneous population of myeloid cells that are essential for maintaining homeostasis and as a first line of innate responders controlling and organizing host defenses against pathogens. Monocyte–macrophage lineage cells are among the most functionally diverse and plastic cells of the immune system. They undergo specific activation into functionally distinct phenotypes in response to immune signals and microbial products. In mammals, macrophage functional heterogeneity is defined by two activation states, M1 and M2, which represent two polar ends of a continuum exhibiting pro-inflammatory and tissue repair activities, respectively. While the ancient evolutionary origin of macrophages as phagocytic defenders is well established, the evolutionary roots of the specialized division of macrophages into subsets with polarized activation phenotypes is less well defined. Accordingly, this chapter focuses on recent advances in the understanding of the evolution of macrophage polarization and functional heterogeneity with a focus on ectothermic vertebrates.

1.1 Introduction

Monocyte–macrophage lineage cells are found across all vertebrate species and play critical roles in homeostasis, wound healing, and immune responses. In addition to providing a first line of defense against pathogens, macrophages also undergo molecular reprogramming in response to microbial-, environmental-, and immune-derived signals that influence their subsequent interactions with various lymphocyte subsets [reviewed in (Biswas and Mantovani 2010; Mantovani et al. 2002; Mosser and Edwards 2008)]. This ability of macrophages to impact the course of immune responses is, in a large part, due to the inherent and adaptable plasticity of these cells. Indeed, monocyte–macrophage lineage cells have long been recognized as highly plastic with polarized populations that differ in terms of effector functions, cell surface receptor expression, and cytokine production

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[reviewed in (Italiani and Boraschi 2014)]. Broadly speaking, two main types of activation or polarization states are defined in mammals: a classically activated/inflammatory (M1) type macrophage and an alternatively activated/regenerative (M2)-type macrophage (Gordon and Martinez 2010; Gordon and Taylor 2005; Mills 2012). From an evolutionary perspective, it is unclear how far this definition of macrophage function stretches outside mammals. However, there are multiple lines of evidence of functional diversification of macrophages in ectothermic vertebrates (Bystrom et al. 2008; Grayfer et al. 2014c; Grayfer and Robert 2014, 2015; McKinney et al. 1986; Rieger et al. 2010). While these functional variations may not strictly adhere to the mammalian paradigms, they imply that the ability of macrophages to adapt their roles to species-specific physiological cues is an evolutionary ancient trait.

Importantly, while the high plasticity of macrophages allows them to modify and reprogram their effector functions in response to immune stimuli, this functional malleability can also be manipulated by invading pathogens. Although macrophage adaptability provides a selective advantage in host resistance to pathogen, this same plasticity can sometimes be exploited and subverted by pathogens to invade the host. Indeed, in mammals, it is not infrequent in an infectious disease setting to find pathology associated with dynamic changes in macrophage activation, where M1 macrophages are associated with initiating and sustaining inflammation and M2 macrophages are associated with either resolution or chronic infection (Cassetta et al. 2011; Herbein and Varin 2010; Labonte et al. 2014; Sang et al. 2015; Shaked et al. 2014). Interestingly, in mouse models, the polarized M1/M2 phenotypes can, to some extent, be experimentally reversed in vitro and in vivo, which makes macrophages interesting targets for immune-modulation and therapeutic applications (Guiducci et al. 2005; Saccani et al. 2006).

As the embryonic origin, lineage commitment and monopoiesis are considered in another chapter of this book, and amphibian myelopoiesis has recently been discussed in detail in (Grayfer and Robert 2016), this chapter will focus on the current understanding of the functional diversification potential of macrophages from an evolutionary perspective. We will particularly discuss comparative approaches aimed at defining macrophage plasticity in genetically distant species in bony fish and amphibians.

1.2 Macrophage Polarization in Mammals

The original definition of mammalian M1 and M2 macrophages is derived from the Th1 and Th2 cytokines associated with their respective polarization and stem from the observation that macrophage activated with either the cytokine interferon gamma (IFN-γ) or lipopolysaccharides (LPS) in mouse strains with T helper type 1 (Th1) or T helper type 2 (Th2) backgrounds differed in their arginine metabolism (Mills et al. 2000). M1-type macrophages utilize inducible nitric oxide synthase (iNOS) to convert l-arginine to l-citruline and nitric oxide (NO) whereas M2

macrophages utilize arginase to convert l-arginine to l-ornithine, which is a precursor for polyamines and proline components of collagen, an important component in tissue repair (Mills 2001). For a detailed review of the immunobiology and regulation of iNOS, see the chapter by Lee at al. Notably, byproducts derived from either the iNOS or arginase pathways inhibit the reciprocal enzymes, thus stabilizing the M1 or M2 macrophage polarization states, respectively (Morris 2009; Munder et al. 1999). In mammals, classical M1 activation is induced by intracellular pathogens, bacterial cell wall components, and hallmark Th1 cytokines such as IFN-γ. This activation results in: (i) the production of an array of pro-inflammatory mediators including interleukin-12 (IL-12), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), interleukin 18 (IL-18), and interleukin 23 (IL-23); (ii) the production of reactive oxygen intermediates (ROI) and nitric oxide synthase-2 (NOS-2/iNOS)-dependent reactive nitrogen intermediates; and (iii) high antigen presenting activity resulting in an effective pathogen-killing phenotype. Comparably, M2 macrophage activity, originally defined as an alternative pathway of macrophage activation characterized by increased mannose receptor activity, is induced by interleukin 4 (IL-4) (Stein et al. 1992). More recent studies have shown that in addition to the prototypical Th2 cytokines IL-4 and IL-13, M2-like activation can be further amplified by fungal pathogens, parasites, immune complexes, components of the complement system (i.e., small proteins that normally circulate as inactive components but can be activated in response to pathogens), apoptotic cells, interleukin 10 (IL-10), and transforming growth factor (TGF-β). M2 macrophages are highly phagocytic, produce immunosuppressive cytokines such as TGF-β and IL-10. These cytokines establish a positive feedback loop that increase M2 macrophage polarization and typically facilitate the resolution of inflammation. However, M2 macrophages can also be reservoirs for intracellular pathogens facilitating chronic infection, aid in the growth of tumors, and cause allergic inflammation (Sica and Mantovani 2012).

The M1/M2 macrophage activation paradigm originally proposed to reflect the Th1 and Th2 nomenclature still requires frequent updates and fine-tuning to take into account the wide functional plasticity and high heterogeneity of macrophage populations as well as to integrate novel findings. One should realize that this M1/M2 paradigm similar to the now revised Th1/Th2 paradigm (including Treg, Th17, Th22, Th9, and T_{FH} cells) represents two polar ends of a spectrum that may not fully take into account all the different activation scenarios. A uniform terminology based on both the tissue source of macrophages and the activation stimuli have recently been proposed (Murray et al. 2014). For example, M2 macrophage responses, depending on the stimuli has lead to the characterization of several M2 subtypes including: M2a (induced by exposure to IL-4 and IL-13); M2b (macrophages activated by immune complexes, Toll-like receptors (TLRs), or apoptotic cells); and M2c (macrophages deactivated by glucocorticoids, TGF-β or IL-10) (Biswas and Mantovani 2010; Mantovani et al. 2004; Martinez and Gordon 2014). While M2a and M2b macrophages drive Th2 responses, M2c cells are primarily involved in immune suppression and tissue remodeling (Murray et al. 2014). It is important to remember that M1/M2 macrophage polarization in vivo is not absolute

and likely reflects a less straightforward and more complex process. Indeed, the list of macrophage subpopulations is still growing and additional subsets of monocyte-derived macrophages are actively investigated including tumor-associated macrophages (TAM), $CD169^+$ macrophages, and most recently TCR^+ macrophages (Allavena et al. 2008a,b; Mantovani et al. 2002; Sica et al. 2002) and [reviewed in (Chavez-Galan et al. 2015)].

An additional challenge in defining activated macrophage subsets comes from species-specific variations. While relatively consistent molecular signatures is found between human and mouse macrophage cell lines, there is a variability in markers used to define M1 and M2 polarization between the two species in vivo (Martinez et al. 2013). For instance, there are no human homologs of the mouse M2 marker chitinase-3-like protein 3 (Chi3l3), also known as Ym1, highlighting the interspecies differences between human and murine macrophages (Raes et al. 2005; Scotton et al. 2005). Nevertheless, the M1/M2 model provides a convenient framework, even though it is probably more accurate to view macrophage polarization as a continuum with overlapping cell surface expressions, cytokine secretions, and transcriptional regulators. In this context, studies aimed at elucidating the functional diversification of macrophages and the mechanisms underlying their activation in other evolutionary distant species may reveal useful understanding how macrophages adapt their function in responses to physiological and microbial cues.

1.3 Evolutionary Conservation of M1/M2-Like Functional Heterogeneity

Phagocytic cells with monocytic morphology are among the most ancestral immune-like cell types existing. We should not forget that the very first description of a macrophage by Élie Metchnikoff in 1905 was from the larvae of starfish, an echinoderm belonging to the class asteroida, a sea invertebrate (Metchnikoff 1905). However, while the evolutionary ancient origin of macrophages is well established, the origin and possible mechanisms governing macrophage functional heterogeneity in nonmammalian species is less well defined. Invertebrates such as insects, nematodes, echinoderms, mollusks, tunicates, or sponges possess numerous types of phagocytic leucocytes, collectively named immunocytes [i.e., hemocyte, coelomocyte, amebocyte, and plasmatocyte, reviewed in (Ottaviani 2011)]. These cells can respond to foreign material by secreting a variety of biologically active pathogen-binding and pathogen-killing substances including NO, reactive oxygen species, and hydrolytic enzymes (Dzik 2014; Franchini et al. 1995; Ottaviani and Franceschi 1997). Such a response is reminiscent of an M1-like phenotype. Conversely, invertebrate immunocytes can also exhibit some M2-like traits including wound-healing activity and to some extent involvement in tissue metabolism. For example, following activation, in the grey slug (*Limax maximus*), hemocytes have been shown to change their phenotype and transition into a collagen-fibrobast-like

phenotype (Franchini and Ottaviani 2000). In addition, gene orthologs encoding some M2 markers such as mannose receptor proteins, chitinase-like proteins, and glycoproteins that share homology with Ym1 are also found in invertebrate phagocytes, which suggests that hemocytes are involved in wound healing and extracellular matrix synthesis (Badariotti et al. 2007; Kirkpatrick et al. 1995; Sricharoen et al. 2005). However, as stated above, microbial infections elicit both M1-like and M2-like transcriptional changes in invertebrate hemocytes, which implies that a strict mammalian type M1/M2 separation is probably lacking in invertebrates [reviewed in (Roszer 2015)].

Besides prototypical M1 and M2 functions, macrophage specialization into mammalian M1- and M2 activation states can, at least partly, be inferred by tracing the molecular evolution of important receptors and effector molecules. As previously mentioned, the mammalian M1/M2 dichotomy is related to the cellular use of arginine (Mills et al. 2000). This amino acid can, through the activity of arginase, be converted into ornithine or alternatively be converted into nitric oxide by the activity of iNOS. The latter reaction makes the macrophage capable of killing invading pathogens, whereas the former is more tuned to tissue repair. Thus, the origin of macrophage specialization into mammalian M1- and M2 types can be considered to stem from two ancient molecular mechanisms: the cytotoxic activity of iNOS and the healing functions of arginase (Dzik 2014). While nitric oxide synthase functions similarly in invertebrates as in vertebrates, nitric oxide primarily serves as a signaling rather than a cytotoxic molecule in invertebrates. Hence, it has been argued that the ability of M1-like macrophages to produce large amounts of nitric oxide in response to microbial infections has emerged with vertebrates. Comparably arginase-1, which since the initial discovery of M2 macrophage activation is considered a prototypic murine M2 marker (Stempin et al. 2010), is ubiquitously found in prokaryote and eukaryotes. From an evolutionary perspective, arginase-1 has been proposed to have primarily been a wound healing protein, transcriptionally induced by proteins of the TGF family (Dzik 2014). However, most microorganisms and invertebrates possess a single arginase gene not involved in the ornithine–urea cycle (Samson 2000). In contrast, two genes encoding distinct, arginase isoforms have been identified in mammals: the cytosolic arginase-1 that is induced by IL-4 and IL-13 and the mitochondrial-associated arginase-2 that is upregulated by IL-10 and LPS (Lang et al. 2002; Munder et al. 1999). Moreover, two arginase genes have been described amphibians (Patterton and Shi 1994) and bony fish (Wright et al. 2004). Thus, based on molecular evidence, arginase, one of the key components driving the molecular mechanisms involved in macrophage polarization, can be traced as far back as bony fish, suggesting that M1/M2-like macrophage specialization arose during early vertebrate evolution.

1.3.1 Bony Fish

In bony fish, the best characterized macrophage phenotype is reminiscent of the mammalian pro-inflammatory M1 state [reviewed in (Hodgkinson et al. 2015)]. Studies have demonstrated that following exposure to various activation stimuli, bony fish macrophages display increased phagocytosis, increased production of reactive oxygen intermediates, elevated expression of inducible nitric oxide synthase (iNOS/NOS2), phagolysosomal acidification, and nutrient deprivation (Grayfer et al. 2014c; Neumann et al. 2000; Rieger et al. 2010). In addition, bony fish macrophages respond as mammalian macrophages to M1-inducing stimuli and microbial challenge by upregulating a prototypic M1 cytokine repertoire including TNFα, IL-1β, interleukin 6 (IL-6), IL-12, interleukin 15 (IL-15), and IL-23 (Arts et al. 2010; Joerink et al. 2006a; Wang and Secombes 2013). While the classical M1-like activation of bony fish macrophages have been relatively well described, the functional occurrence of alternatively activated/M2 macrophages has proven more challenging to define. Efforts to characterize M2-like macrophages in fish have largely focused on the biology of typical M2 stimuli, the best characterized of which being interleukin IL-4 and IL-13. To date, two genes have been identified in bony fish that share homology with both the mammalian IL-4 and IL-13 cytokines (IL-4/13A and IL-4/13B) (Ohtani et al. 2008; Wang et al. 2016). Using a rainbow trout (*Oncorhynchus mykiss*) in vitro head kidney leucocyte culture system (in fish, myelopoiesis occurs in the head kidney), recombinant forms of both IL-4/13A and IL-4/13B were shown to be anti-inflammatory via upregulation of IL-10 and downregulation of IL-1β and IFN-γ (Wang et al. 2016). However, whether or not these cytokines directly act to polarize macrophages towards an M2-like state remains to be determined.

As previously stated, another important hallmark of M2 activation is arginase activity. Using an in vitro macrophage model in the common carp (*Cyprinus carpio L.*), it was demonstrated that macrophages derived from head kidney leukocytes could be polarized into two different states by incubation with either dibutyryl cyclic adenosine mono phosphate (cAMP) or LPS. In this system, cAMP treatment resulted in highly upregulated arginase-2 gene expression, indicative of an M2-like phenotype (Joerink et al. 2006b,c). This is intriguing since cAMP stimulation has been shown to transform human M1 macrophages into resolution-phase macrophages (rMs) (Bystrom et al. 2008). These rMs exhibit an M2-like phenotype but with elevated M1 cell markers such as nitric oxide synthase (iNOS) and, thus, are neither classical M1 nor M2 macrophages. Rather, rMs have a role in restoring tissue homeostasis following resolution of a inflammatory response. Finally, glucocorticoids, immune complexes, and IL-10 have been shown to be immune-suppressive on bony fish macrophages.

More recently, M1-like and M2-like polarization was demonstrated in vivo using a transgenic zebrafish (*Danio rerio*) larval model (Nguyen-Chi et al. 2015). This model uses TNFα gene expression as a marker for M1 macrophages and takes advantage of double transgenic fish lines in which macrophages express mCherry

under the control of the macrophage-specific *mpeg1* promoter and eGPF under the control of the *tnfa* promoter Tg (*mpeg1:mCherryF*/*tnfa*:eGFP-F) in combination with wound-induced inflammation. By examining the gene expression profiles of *tnfa*$^{-}$ and *tnfa*$^{+}$ macrophage populations isolated during early and late phases of inflammation, well-known markers of mammalian M1 macrophages including TNFα, IL1-β, and IL-6 were expressed in the *tnfa*$^{+}$ macrophages, whereas *tnfa*$^{-}$ macrophages expressed TGF-β, C-C chemokine receptor type 2 (CCR2), and C-X-C chemokine receptor type 4 (CXCR4), which are markers of M2 activation in mammals (Beider et al. 2014; Hao et al. 2012; Mantovani et al. 2002; Martinez et al. 2006). Of note, neither *Arginase 1* (*Arg1*) nor IL-10 expression was detected in zebrafish *tnfa*$^{-}$ macrophages. Collectively, these observations strongly argue for an evolutionary conservation of both M1-like and M2-like activation states in zebrafish. However, it is important to remember that both similarities and differences have been documented for the regulation of bony fish macrophage antimicrobial defenses, as compared to what has been described in mammals.

1.3.2 Amphibians

Anuran amphibians like *Xenopus* present the peculiarity of developmentally distinct macrophage populations in tadpoles and adults frogs, which can be further polarized into distinct functional subsets. Tadpole and adult macrophages elicited by intraperitoneal injection with heat-killed *E. coli*, and subsequently allowed to adhere in vitro in culture plates, are morphologically distinct. Compared to adult macrophages, a subset of tadpole macrophages is enlarged with a highly vacuolated morphology (Grayfer et al. 2012). Although hematopoiesis in *Xenopus* is localized to the subcapsular liver, macrophage precursors in X. laevis, unlike other vertebrates, are derived from the bone marrow [(Grayfer and Robert 2013) and reviewed in (Grayfer and Robert 2016)]. Since the bone marrow is absent in tadpoles, it is currently unknown how tadpole macrophage development and differentiation occurs.

Although M1/M2 is not fully defined in *Xenopus*, there is clear evidence in both tadpole and adult X. laevis of functional specialization driven by the two different colony stimulating factor-1 receptor (CSF-1R) ligands: colony-stimulating factor-1 (CSF-1) and interleukin-34 (IL-34) (Grayfer and Robert 2014, 2015). Across most vertebrate species, the survival, proliferation, differentiation, and functionality of cells of the monocytic lineage require binding of CSF-1 to its receptor CSF-1R (Dai et al. 2002), which is a marker for committed myeloid precursors and phagocyte populations (Guilbert and Stanley 1980; Lichanska et al. 1999; Tagoh et al. 2002). Although CSF-1 and CSF-1R genes display relatively low sequence conservation across evolutionary distant species, important hallmark sequence features as well as the intracellular signal-binding sites are highly conserved suggesting a shared function [(Garceau et al. 2010; Grayfer et al. 2014b; Grayfer and Robert 2013; Hanington et al. 2007; Wang et al. 2008) and reviewed in (Grayfer and Robert

2016)]. In support of the conserved function of CSF-1R, adult X. laevis bone marrow cells have been shown to respond to X. laevis recombinant CSF-1 and differentiate into primarily large mononuclear cells with characteristic macrophage morphology (Grayfer and Robert 2013). Similarly, bony fish CSF-1 is a strong inducer of macrophage growth and differentiation (Grayfer et al. 2009; Hanington and Belosevic 2007). Interestingly, the interleukin-34 (IL-34) cytokine, which is found across evolutionary distant species also binds to and activates CSF-1R in mammals resulting in the initiation of distinct biological activity and signal activation (Chihara et al. 2010; Liu et al. 2012; Wei et al. 2010). It is becoming increasingly evident that in X. laevis, the two types of macrophages elicited by either CSF-1 or IL-34 cytokines have distinct polarizing roles during infections both in tadpoles (Grayfer and Robert 2014) and adult frogs (Grayfer and Robert 2015) as discussed in the next section.

1.3.2.1 Macrophage Polarization During X. laevis Antiviral Responses

There is ample evidence implicating amphibian macrophages as important components in evasion, persistence, and dissemination during infections with the ranavirus, frog virus 3 (FV3), which is a large pox-like icosahedral dsDNA virus [reviewed in (Chinchar et al. 2011; Grayfer et al. 2012) and more recently in (Grayfer and Robert 2016)]. Recent findings indicate that FV3 infects and likely uses macrophages to persist and disseminate throughout the host, suggesting that macrophage functions are modulated by the virus (Morales et al. 2010; Robert et al. 2007). In general, tadpoles are more susceptible and succumb to FV3 infections within 1–2 months, whereas adults are more resistant and typically clear the infection within a few weeks (Bayley et al. 2013; De Jesus Andino et al. 2012; Grayfer et al. 2014a; Hoverman et al. 2010).

Notably, the administration of X. laevis recombinant CSF-1 increases the susceptibility of X. laevis tadpole to FV3 infections (Grayfer and Robert 2014). In contrast, recombinant IL-34 increases tadpole resistance to FV3 infection (Grayfer and Robert 2014). As previously suggested (Grayfer and Robert 2014, 2015), this is possibly due to a CSF/IL-34-mediated expansion and biased polarization towards macrophage subpopulations with distinct antimicrobial properties. Consistent with these observations, in vitro studies have demonstrated that IL-34-elicited peritoneal macrophages express higher baseline levels of type I interferon, which following FV3 infection was further enhanced compared to CSF-elicited peritoneal macrophages (Grayfer and Robert 2014). IL-34-elicited peritoneal macrophages also express elevated levels of the NADPH oxidase components $p67^{phox}$ and $gp91^{phox}$ and display an increase in the major histocompatibility complex class I and II as well as β2-microglobulin gene expression. This is consistent with an enhanced antigen presentation capacity, which would contribute to enhance T cell-mediated anti-viral response (Grayfer and Robert 2014).

Similar studies focused on adult peritoneal macrophages have confirmed that X. laevis possess functionally distinct macrophage populations (Grayfer and Robert

Table 1.1 Characteristics of CSF-1- and IL-34-derived X. laevis peritoneal macrophages

	Effector molecules expressed	Increased effector functions	Proposed function	References
CSF-1-derived	iNOS[a]	Phagocytosis, NO production Microbicidal activity	Antibacterial	Grayfer and Robert (2014, 2015)
IL-34-derived	Arginase-1 NADPH oxidase Type I IFN[b]	ROS production Antiviral activity	Antiviral	Grayfer and Robert (2014, 2015)

[a]Significantly higher iNOS expression in CSF-1-derived than IL-34-derived adult peritoneal macrophages
[b]Significantly higher Type I IFN gene expression response in tadpole but not adult IL-34-derived peritoneal macrophages compared to respective CSF-derived peritoneal macrophages

2015). Similar to tadpoles, IL-34-elicited adult peritoneal macrophages exhibit more potent antiviral activity against FV3 than CSF-elicited macrophages, which is indicated by a higher gene expression of NADPH oxidases components, a greater respiratory burst response (i.e., release of reactive oxygen species) and reactive oxygen production (Grayfer and Robert 2015) All these features are consistent with a M1-like phenotype. Contrasting with the classical M1 mammalian definition, however, IL-34 elicited macrophages also possess an elevated arginase-1 gene expression, which in mammals and fish is typically associated with the alternatively activated M2 phenotype. Characteristics of CSF-1- and IL-34-elicited X. laevis macrophage are summarized in Table 1.1.

1.3.2.2 Macrophage Polarization During X. laevis Antibacterial Responses

As in the case of viral pathogens, macrophages that are essential immune effector cells are also used by certain intracellular bacteria to survive and thrive. An example in humans highlighting this paradoxical role of macrophages is *Mycobacterium tuberculosis* (M*tb*), the causative agent of tuberculosis (TB). M*tb* preferentially infects macrophages, replicates and persists inside the cell, and causes chronic infections. A hallmark feature of the immune response to M*tb* infections is the formation of compact aggregates or granulomas primarily made up of blood-derived macrophages and epithelioid cells (i.e., activated macrophages that often merge together via tightly interdigitated cell membranes that link adjacent cells) surrounded by additional immune cells including neutrophils, dendritic cells, T cells, and B cells [reviewed in (Silva Miranda et al. 2012)]. It has been postulated that granulomas are formed in an attempt to wall off and sequester Mtb. However, granulomas also provide mycobacteria with a niche in which it can modulate the immune response, survive over long periods of time, and under certain conditions reactivate and cause clinic disease (Adams 1976; Martin et al. 2016). This ability of Mtb to survive inside macrophages is postulated to result in part from the M*tb*s

ability to subvert pro-inflammatory M1 functions (Cronan and Tobin 2014; Huang et al. 2015; Marino et al. 2015) To understand how M*tb* can modulate macrophage polarization and functions especially during early stage of granuloma formations, nonmammalian alternative animal models have proven valuable (O'Toole 2010). For example, important insight into the dynamic structures of granulomas has been gathered from studies using *Mycobacterium marinum* (M*m*) infected zebrafish models (Cronan and Tobin 2014; Davis et al. 2002; Davis and Ramakrishnan 2009; Meijer 2016; Meijer et al. 2005; Stinear et al. 2008). M*m* is a natural pathogen of ectothermic vertebrates causing a systemic disease with formation of macrophage aggregates and containment of bacteria in granulomas that show strong similarity with human TB granulomas. Studies in zebrafish not only confirmed the importance of macrophage-mediated phagocytosis and initial pro-inflammatory signaling in controlling M*m* growth, but also provided a novel insight into host macrophage–mycobacteria interaction (Cronan and Tobin 2014; Davis et al. 2002; Davis and Ramakrishnan 2009; Meijer et al. 2005). Notably early granuloma formation, which was thought to be a relatively static host defense mechanism, was shown in zebrafish to be a dynamic structure actively utilized by M*m* to, via the M*m* virulence factor RD-1, recruit nascent macrophages to the site of the primary granuloma where they phagocytose the bacillus (Davis and Ramakrishnan 2009). Subsequently newly infected macrophages depart the primary granuloma, initiate new granulomas at distal locations, and thus facilitate M*m* dissemination (Cronan and Tobin 2014; Davis et al. 2002; Davis and Ramakrishnan 2009; Meijer et al. 2005). In addition, using the zebrafish model, it has been suggested that M*m* manipulate macrophage recruitment to preferentially recruit a subpopulation of iNOS-deficient macrophages. This would selectively enhance Mm phagocytosis by macrophages with reduced bactericidal activity and thus provide a niche for bacterial growth (Cambier et al. 2014).

Moreover, genetically engineered X. laevis transgenic lines expressing fluorescent reporter genes in macrophages are likely to prove useful for further in vivo characterization of macrophage activity and polarization during a complex infection in living animals. In addition, unlike zebrafish, T cells develop within 2 weeks of age in X. laevis tadpoles. Furthermore, the majority of these T cells are so-called innate T cells expressing semi-invariant T cell receptors that are restricted by MHC class-I-like molecules [reviewed in (Edholm et al. 2014; Robert and Edholm 2014)]. Specifically, deep-sequencing has revealed that 80% of CD8 intermediate and CD8 negative T cells express 6 invariant TCRα rearrangements (Edholm et al. 2013). The polarization potential of one or more of these iT cell population on macrophages open new avenues of investigation. Indeed, preliminary evidence using loss-of-function reverse genetic combined with transgenesis has identified an iT cell subset critical for anti-M*m* host resistance (i.e., transgenic tadpoles lacking this iT cell population are more susceptible to M*m* infection; Edholm, Rhoo and Robert, unpublished).

As in zebrafish, several transgenic lines with different subsets of myeloid lineages labeled with different colored fluorescent proteins have been characterized (Paredes et al. 2015). These lines include: a *xLurp1*:EGFP Tg line in which myeloid cells (e.g., granulocytes and monocytic leukocytes) express EGFP under the *xLurp* (Ly-6/uPAR-related protein) promoter (Paredes et al. 2015; Smith et al. 2002); a *xmpeg*:mCherry Tg line in which only mononuclear phagocytes (i.e., mainly macrophages) express the red fluorescence label mCherry under the zebrafish macrophage-specific *mpeg* promoter (Ellett et al. 2011); and a double *mpeg1*:mCherry and x*lurp1*:GFP line (Paredes et al. 2015).

Using this double transgenic line in combination with fluorescently labeled M*m*, the diversity and plasticity of X. laevis macrophage subset during infection has begun to be further examined in vivo in adult and tadpoles (Fig. 1.1). Using intravital imaging, it is possible to visualize in real time the migration, accumulation, and granulomatous formation in response to M*m* infection (Rhoo and Robert, unpublished data). With regard to IL-34/CSF driven macrophage polarization during M*m* infection in *Xenopus*, distinct expression kinetics of IL-34 and CSF gene have been observed in pilot experiments in adult frogs. Notably, IL-34 is highly expressed during the early stages of infection peaking at 6 dpi followed by decreased expression at later time points. In contrast, CSF-1 gene expression continues to increase until 12 days post-M*m* infection suggesting a polarization from M1-like to M2-like phenotype during M*m* infection highlighting the importance of macrophage effector choices in the context of M*m* infection progression. Although still preliminary, these observations further implicate CSF-1 and IL-34 as important factors in macrophage polarization and function during mycobacteria infection (Table 1.1).

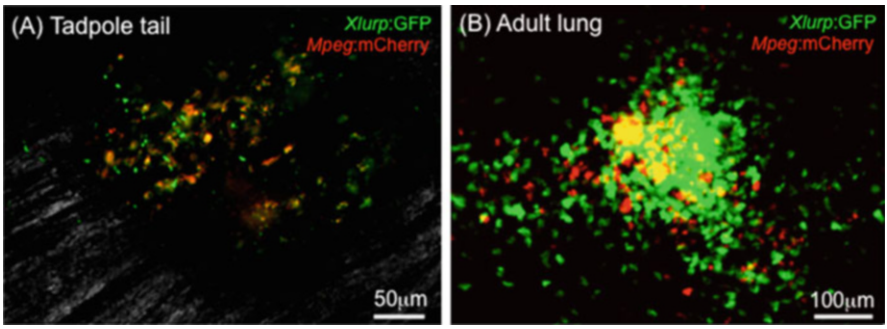

Fig. 1.1 Visualization of macrophage involvement in granuloma formation during Mm infection in *xlurp*:GFP/*xmpeg*:mCherry double transgenic X. laevis. (**a**) Recruitement and accumulation of lurp+/mpeg+ (*red-orange*) macrophages at the site of infection in the tadpole's tail 8 days after *Mm* infection. The infection was done by intramuscular injection of 1000 forming unit (CFU) in a volume of 100 nl in the middle section of the tail of a 3 weeks old tadpole. (**b**) Small granuloma-like accumulation of lurp+/mpeg- leukocytes (*green*) and lurp+/mpeg+ macrophages (*red-orange*) in the lung of a young adult 19 days after *Mm* infection by intraperitoneal injection of 1×10^6 CFU in a volume of 100 μl. Images were taken on unfixed whole mount organ for adult and live tadpole under narcosis using a Leica DMIRB inverted fluorescence microscope

Concerning the role of CSF and IL-34 in macrophage polarization, it is noteworthy that CSF-1 can alter the magnitude of M1/M2 polarized phenotypes in mammals (Verreck et al. 2004, 2006). For example, human macrophages cultured in presence of recombinant CSF-1 poorly respond to LPS ± IFNγ stimulation. However, while these CSF-1 treated cells are unable to generate the pro-inflammatory cytokines, IL-12 or IL-23, they can produce significant amounts of IL-10 in response to the same stimuli (i.e., LPS ± IFNγ). Thus, although CSF-1 stimulation alone does not recapitulate a full M1/M2 phenotype when compared with prototypic polarizing stimuli (e.g., IFNγ, TLRs, IL-4, IL-13, etc.), CSF-1 stimulation has impacts on macrophage polarizing sensitivity. These studies suggest that CSF-1 predisposes monocyte–macrophage to exhibit a differential M2 phenotype (Verreck et al. 2004, 2006). Notably, CSF-1-derived human monocytes are more susceptible to infection with an attenuated strain of *Mycobacterium bovis* (Bacille de Calmette et Guérin), the causative agent of tuberculosis in cattle, as determined by increased phagocytosis and enhanced bacterial outgrowth (Verreck et al. 2004, 2006). Although the modulating potential and putative suppressive role of CSF-1 in macrophage polarization is partially recapitulated in mice (Fleetwood et al. 2007) and to some extent in *Xenopus*, in bony fish CSF-1 stimulation appears to skew macrophages towards an M1-like state (Grayfer et al. 2009) and reviewed in (Hodgkinson et al. 2015).

Interestingly, the single CSF-1 gene in mammals and birds exhibits alternatively splicing (Garceau et al. 2010; Manos 1988; Rettenmier and Roussel 1988), whereas in amphibians the single CSF-1 gene does not appear to be alternatively spliced (Grayfer and Robert 2013) and bony fish possess two distinct CSF-1 genes (Wang et al. 2008). It is presently unknown whether the distinct fish molecules encoded by these two CSF-1 genes have distinct biological roles and, whether, as previously suggested (Grayfer and Robert 2016) these gene products may recapitulate the mammalian CSF-1 splice variants.

Thus, it appears that bony fish and amphibian macrophages, similar to their mammalian counterparts, exhibit a variety of functional roles and an ability to become polarized towards either an M1-like inflammatory type or an M2-like resolution functional state (summarized in Table 1.2). However, more extensive comparative research will be required for elucidating mechanisms governing macrophage functional heterogeneity and for understanding macrophage evolution. Also, given the heterogeneity in macrophage populations, it is likely that the evolution of macrophage polarization is more complex, reflecting the species adaptations, the environment and, the source of pathogenic stimuli. For example, anuran amphibians not only exhibit two quite distinctive developmental stages: tadpoles and adult frogs, but these life stages also exhibit different antigen receptor repertoires for both B and T cells and occupy different ecological niches (e.g., different diets and biotopes) that are likely confronted to different pathogens. As such tadpole and adult frog macrophages are likely exposed to different stimuli.

Table 1.2 Summary of prototypical M1 and M2 macrophage functions and hallmark genes

	M1-like characteristics				M2-like			
Animal taxa	Antimicrobial activity	NO, ROI iNOS	M1 polarizing cytokines (IFNγ TNFα)	M1-cytokine profiles[a]	Wound healing	Arginase[b]	M1 polarizing cytokines (IL-4, IL-13)	M1-cytokine profiles[c]
Invertebrates (insects, snails)	+	+	?	?	+	+ Arginase-2	–	?
Teleost (Cyprinids, salmonids)	+	+	+	+	+	+ Arginase-1/2	+[d]	+
Amphibians (*Xenopus*)	+	+	+	+	+	Arginase-1/2	+[e]	+
Mammals (Human, mice)	+	+	+	+	+	Arginase-1/2	+	+

[a]Up-regulation of pro-inflammatory cytokines in response to microbial challenge, including TNF-α, IL-1β, IL-12 and IL-23, has been demonstrated in teleost and amphibians

[b]Arginase is encoded by a single gene in invertebrates, whereas in vertebrates two genes code for the cytosolic arginase-1 and the mitochondria arginase-2 (postulated to represent the ancestral gene). Mammalian M2 macrophage express arginase-1, while alternative macrophage activation in teleost (carp) coincides with increased arginase-2 transcript levels

[c]M2-type cytokines such as IL-10 and TGF-β have been described in teleost and amphibians

[d]In teleost fish, two genes share homology with both IL-4 and Il-13 (IL4/13A and IL4/13B)

[e]To date in *Xenopus,* a single gene shares homology with both IL-4 and IL-13 (IL4/13)

1.4 Conclusion

Functional heterogeneity and adaptable plasticity are hallmarks of monocyte–macrophage lineage cells, highlighting the essential roles of these cells in maintaining homeostasis, as well as effector functions during pro-inflammatory and anti-inflammatory immune responses. Similar to their mammalian counterparts, macrophages of ectothermic vertebrates are now recognized to have the ability to adapt their functional roles to species-specific physiological cues suggesting that macrophage functional polarization towards distinct activation states is an evolutionary ancient trait. However, although broadly defined M1-like and M2-like macrophages have been demonstrated in nonmammalian species, this terminology covers an array of functionally disparate groups of macrophages. As such the full spectrum of macrophage activation, polarization, and functions in vivo is less straightforward and not strictly adhering to their mammalian counterparts. In addition, although T and B cell-deficient mice do possess the potential for macrophage polarization (Mills et al. 2000), the immune system consists of a spectrum of immune cell populations clearly influencing and being influenced by macrophage polarization. Indeed, it is likely that macrophages encounter both M1-like and M2-like polarizing stimuli simultaneously within an inflamed tissue microenvironment, which may in part explain the wide spectrum of macrophage-activated phenotypes in vivo. Furthermore, it is well recognized that depending on anatomical and physiologic settings, macrophages exhibit different capacities for polarization. Thus, further research into the molecular cues and mechanisms regulating mammalian and nonmammalian macrophages will further the understanding of macrophage functional regulation.

In this regard, the growing genetic resources in particular the availability of ectothermic vertebrates transgenic lines combined with live-imaging techniques will be useful in the further deciphering of macrophage polarization. Indeed, nonmammalian animal models such as zebrafish and *Xenopus* present several attractive features for in vivo studies and intravital microscopy. Because of external fertilization, early developmental stages are accessible to experimentation. In addition, zebrafish larvae and *Xenopus* tadpoles have a relatively small size, are transparent, and do not require temperature control or extensive aseptic conditions. All these attributes are convenient for intravital microscopy. The availability of inbred lines allowing adoptive cell transfer as well as transgenic lines with fluorescently labeled macrophage and other immune cell populations further empower these nonmammalian animal models. Owing to the additional possibilities offered by loss-of-function approaches using genome editing technology, ectothermic vertebrates models are in position to significantly advance our understanding of the plasticity and “raison d’être” of macrophage activation.

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Chapter 2
Development and Functional Differentiation of Tissue-Resident Versus Monocyte-Derived Macrophages in Inflammatory Reactions

Paola Italiani and Diana Boraschi

Abstract Mononuclear phagocytes are key cells in tissue integrity and defense. Tissue-resident macrophages are abundantly present in all tissues of the body and have a complex role in ensuring tissue functions and homeostatic balance. Circulating blood monocytes can enter tissue both in steady-state conditions, for helping in replenishing the tissue-resident macrophage pool and, in particular, for acting as potent effector cells during inflammatory events such as infections, traumas, and diseases. The heterogeneity of monocytes and macrophages depends on their ontogeny, their tissue location, and their functional programming, with both monocytes and macrophages able to exert distinct or similar functions depending on the tissue-specific and stimulus-specific microenvironment. In this short review, we will review the current hypotheses on tissue-resident macrophage ontogeny and functions, as compared to blood-derived monocytes, with a particular focus on inflammatory conditions.

2.1 Introduction

In 1908 the Nobel Prize in Physiology and Medicine was awarded jointly to Paul Ehrlich and Elie Metchnikoff "in recognition of their work on immunity," for their contribution in defining what is today known as adaptive and innate immunity, respectively. In the following century, studies on adaptive immunity boomed, leading to paramount discoveries in the field, while innate immunity was almost forgotten. It is only in the last few years that new highlights into macrophage biology have reverted the trend leading to the recognition of the important role of macrophages not only within the immune response but also in several physiological and pathological processes within the body. An increasing amount of evidence redefines macrophages not only as cells of the innate immune system designated to

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eliminate invading pathogens but also as polyfunctional and plastic cells that define and preserve the tissue integrity at steady state and restore homeostasis upon disturbances. This evidence encompasses their origin (not only from adult bone marrow but also from embryonic hematopoiesis) (Hoeffel and Ginhoux 2015), their heterogeneity in different tissues (they have specific specializations and roles depending on the tissue where they are located) (Gordon and Taylor 2005; Gordon et al. 2014), their capacity to acquire different functional phenotypes (M1/M2 polarization: they are responsible for triggering an inflammatory reaction and afterward of driving its resolution, tissue repair, and remodeling) (Gordon and Taylor 2005; Biswas and Mantovani 2010), and their ability to react differently upon a second challenge (tolerant and trained responses, also known as innate memory) (Netea et al. 2015). Thus, the role of macrophages has changed from sentinels of the body or "trash disposal units" serving at the bequest of T and B cells, to main "conductors" and effectors of the inflammatory response, and to immune memory cells, able to remember (albeit not specifically) previously encountered stimuli. All these new concepts on monocytes/macrophages are revolutionary and will force the researchers to rewrite the textbooks of immunology, finally giving to macrophages the attention they deserve.

What was defined as the reticuloendothelial system (that included all cells with phagocytic capacity) by K.A.L. Aschoff in 1924 (Aschoff 1924) in the 1970s was redubbed the "mononuclear phagocyte system (MPS)," excluding fibroblasts and endothelial cells and including monocytes, all types of macrophages, and dendritic cells (DCs). After a long and hot discussion on the origin of macrophages, the position of Ralph van Furth prevailed that all tissue-resident macrophages are derived from bone marrow or bone marrow-derived blood monocytes (van Furth et al. 1972). Thus, despite a number of studies challenging this position, until few years ago, the common knowledge was that tissue macrophages derive from monocytes, which terminally differentiate in the tissue and are unable to proliferate. The discovery of an embryonic origin of tissue-resident macrophages and of their capacity of in situ self-renewal without loss of their differentiated cellular identity is now well established and generally accepted (Jenkins et al. 2011; Gentek et al. 2014). Amidst new evidence on macrophage biology, this is the one that raises the need of understanding of the characteristics of tissue macrophages as compared to monocytes (that are no longer simple precursors of tissue macrophages), the intrinsic differences between these cell types, and their relative involvement in building and maintaining tissue homeostasis in normal conditions and during an inflammatory reaction.

Without going in detail of the aforementioned aspects (ontogeny, heterogeneity, polarization, memory), which are excellently reviewed elsewhere (Netea et al. 2015; Gentek et al. 2014; Gomez Perdiguero and Geissmann 2016; Murray 2017), this review will summarize the recent advances in our understanding of the origin of tissue macrophages and of their differences from monocytes and monocytes-derived macrophages.

Blood circulating monocytes can be recruited into a tissue in different circumstances. In physiological conditions, they can enter a tissue for replenishing the pool

of resident macrophages that maintain homeostasis. In pathological events, they are recruited for fighting an infection, for restricting the growth of a tumor, or for repairing damage. Once monocytes migrate into a tissue, then, by definition, they become macrophages. Although some investigators use the term tissue monocytes, we support the opinion that the term monocyte should be restricted to cells in the blood compartment and also in the bone marrow and spleen, given that these are reservoirs that can replenish the blood monocyte pool (Ziegler-Heitbrock 2015). Monocytes recruited into the tissue are often referred to as bone marrow-derived macrophages, monocyte-derived macrophages (moMϕ), inflammatory monocytes/macrophages, nonresident macrophages, peripherally derived inflammatory macrophages (newly differentiated macrophages within inflammatory lesions), and so on. Throughout the chapter when we mention monocytes, we will refer to the bone marrow-derived and circulating monocytes (not yet activated and differentiated into macrophages in the tissue), while when we mention moMϕ, we will refer to the bone marrow- and monocyte-derived cells newly recruited in the tissue, in order to distinguish them from tissue-resident macrophages (trMϕ) originating in the embryo and also known as embryonic-derived macrophages.

2.2 Heterogeneity of Circulating Monocytes and Tissue-Resident Macrophages

Historically, the MPS includes monocytes, DCs, and macrophages, distinguished on the basis of functional and phenotypic features (van Furth et al. 1972). A number of issues have complicated the picture leading to much confusion regarding the definition of these cell types and their related subsets. First, the phenotypic markers proposed for the identification often are not unique, and many functions are shared/overlapping between cell types (Hume 2008; dos Anjos Cassado paper 2017). Second, the markers used for defining a particular cell or cell subset are not always consistent between mice and humans (Reynolds and Haniffa 2015; Ziegler-Heitbrock 2014). Third, many trMϕ are of embryonal origin (see below) as opposed to monocytes and DCs that originate from adult hematopoietic stem cell (HSC)-derived progenitors, although there are no markers that can discriminate them uniquely. In order to understand which phenotypical and functional differences distinguish these cell types and to avoid confusion due to the aforementioned issues, a brilliant and fully sharable unified nomenclature has been recently proposed, based primarily on the ontogeny of myeloid cells and secondarily on their location (Guilliams et al. 2014).

Without going into DCs and the hot debate about their origin and relation to monocytes and macrophages (Hume 2008; Geissmann et al. 2010), the focus of this section will be on monocytes and trMϕ. Monocytes are the cells of the myeloid lineage that circulate in the blood stream, while macrophages are the myeloid cells that colonize the tissues throughout the body. Monocytes are generated during adult

hematopoiesis from HSC progenitors in the bone marrow (Ginhoux and Jung 2014), while macrophages originate predominantly during embryonic hematopoiesis, from progenitors in the yolk sac (YS) and fetal liver (FL) (Epelman et al. 2014a; Wynn et al. 2013). The ontogeny/origin of macrophages will be described in the next section. Both monocytes and macrophages are involved in host defense against infections, in maintaining tissue homeostasis and development (Gordon et al. 2014; Wynn et al. 2013), in initiation and resolution of the inflammatory reaction (Italiani and Boraschi 2014), in sensing tissue damage and orchestrating tissue repair and remodeling (Mantovani et al. 2004), and in disease progression (e.g., cancer; Noy and Pollard 2014). While macrophages are already present in tissues, blood monocytes are recruited into the tissue mainly in response to a dangerous event (e.g., damage, infection, or disease) and are the main effectors of the inflammatory reaction. In some cases, monocytes can enter a tissue for homeostatic reasons, acting as cell reservoir for steady-state maintenance of the resident macrophage population (e.g., for dermis and intestine macrophage repopulation - Tamoutounour et al. 2013; Varol et al. 2015). When the challenges (infections or damages) are limited and weak, trMϕ can directly exert defensive functions. However, in order to successfully handle a vast and significant danger, trMϕ can locally proliferate, to increase their number, but their main role is that of initiating the recruitment of inflammatory monocytes from blood.

The advent of polychromatic flow cytometry has enabled the characterization/identification of distinct monocyte and macrophage subsets, demonstrating that both monocytes and macrophages are actually heterogeneous populations. In mice, two different monocyte subsets have been identified, based on the expression of a GPI-anchored surface glycoprotein (Ly6C) and two chemokine receptors (CX3CR1 and CCL2), the $Ly6C^{high}CCR2^{+}$ and the $Ly6C^{low}CX3CR1^{+}$ subsets. Both monocyte subsets can infiltrate peripheral tissues under appropriate stimuli. $Ly6C^{high}$ cells are essential for the inflammatory response, while $Ly6C^{low}$ cells are able to patrol the vascular endothelium and survey its integrity (Auffray et al. 2007). They also seem able to extravasate rapidly into tissues before the arrival of $Ly6C^{high}$ inflammatory monocytes (Sumagin et al. 2010), although their extravasation into tissue is apparently a rare event (Auffray et al. 2007). A number of recent reviews describe the functions and relationship of these two populations (Ziegler-Heitbrock 2015; Ginhoux and Jung 2014; Gutknecht and Bouton 2014; Sprangers et al. 2016). Evidence suggests that $Ly6C^{high}$ monocytes give rise to $Ly6C^{low}$ monocytes, both in the bone marrow and in the circulation (Yona et al. 2013; Hettinger et al. 2013). In humans, three different subsets of blood monocytes are identified based on the relative expression of the surface markers CD14 and CD16: $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate), and $CD14^{+}CD16^{++}$ (nonclassical) monocytes. The functions and the differences between these three subsets are still controversial (Ziegler-Heitbrock 2007; Wong et al. 2012). The classical and nonclassical human monocytes are homologous to their counterparts in mice, $Ly6C^{high}$ and $Ly6C^{low}$, respectively. It seems that classical monocytes are inflammatory and phagocytic, even if it seems that the intermediate are mainly involved in various inflammatory

diseases (Wong et al. 2012; Fingerle et al. 1993) and have been shown to be of prognostic relevance in cardiovascular diseases (Rogacev et al. 2012).

Macrophages are present in all tissues, and their different phenotypes and functional specializations in specific tissue microenvironments explain their heterogeneity (Gordon and Taylor 2005; Epelman et al. 2014a, b; Haldar and Murphy 2014). The heterogeneity of trMϕ is the result of the combination of tissue-identity signals, which promote their local differentiation, and the availability of functional demand signals, which induce different functional phenotypes of the macrophages. In this way, the overall phenotype of trMϕ is perfectly tailored to the needs of the tissue in which they reside, as an integration of stable and irreversible differentiation and stable and reversible polarization programs. Recently, this matter has been described in detail in an excellent review (Okabe and Medzhitov 2016). Thus, macrophages take different names according to their tissue location and specialized functions. For example, bone macrophages are known as osteoclasts and are highly specialized in bone resorption (Boyle et al. 2003). In the lung alveoli, they are known as alveolar macrophages and are responsible for recycling of surfactant molecules (Hussell and Bell 2014). In the brain, trMϕ are known as microglia and sustain the brain development through trophic functions (synaptic pruning, regulation of neuronal precursor numbers, and production of neurotrophic factors) (Schafer et al. 2012; Parkhurst et al. 2013). Liver macrophages are known as Kupffer cells and support the tolerogenic milieu existing within the liver, ensure protection during infections, and maintain iron homeostasis (Eckert et al. 2015). In the skin, they are known as Langerhans cells (LCs) and are involved in immune functions and epidermis/skin homeostasis (Collin and Milne 2016). In the spleen, red pulp macrophages are involved in the processing of heme and iron from senescent erythrocytes (Kohyama et al. 2009), while macrophages in the splenic marginal zone are important for the capture of blood-borne antigens (Aichele et al. 2003) and for the proper positioning of B cells (Karlsson et al. 2003). Macrophages in the bone marrow have a crucial role in the retention of hematopoietic stem cells (HSCs) in the bone marrow (Chow et al. 2011). Intestinal macrophages in the *muscularis mucosa* engage in crosstalk with surrounding muscle cells and enteric neurons to ensuring normal intestinal peristalsis (Muller et al. 2014).

Different gene expression profiles, transcriptional regulatory pathways, and enhancer activities, all driven by the local microenvironment, underlie tissue-specific macrophage identities and specialized functions. All these aspects linked to transcriptional and epigenetic programs are excellently reviewed elsewhere (Romanoski et al. 2015; Glass and Natoli 2016; Amit et al. 2016; Lavin et al. 2014; Lavin et al. 2015; Gosselin et al. 2014).

2.3 Origin of Macrophages

Hematopoiesis is the process by which lymphoid and myeloid lineage cells of the blood are formed, through a tightly regulated stepwise process involving several progenitor cells (De Kleer et al. 2014). Hematopoiesis occurs in several temporally and spatially regulated waves during the embryonic development ("primitive hematopoiesis") in the YS and culminates with the generation of HSCs in the FL before birth and in the bone marrow (BM) after birth and during adulthood ("definitive hematopoiesis"). An exhaustive description of hematopoiesis and of the ontogeny of monocytes and trMϕ is beyond the aim of this review. However, the section below will summarize the key steps of the development of mononuclear phagocytes referring to definitive/adult hematopoiesis as the main source of circulating monocytes and to primitive/definitive/fetal hematopoiesis as the main source of trMϕ.

2.3.1 Monocytes and moMϕ Development: Definitive/Adult Hematopoiesis

Lineage-committed mononuclear phagocytes, i.e., circulating monocytes and moMϕ, are generated primarily in the bone marrow from tightly regulated differentiation of HSCs to progressively more committed cell population. High levels of the transcription factor c-Myc induce the differentiation of HSCs toward more committed progenitors (Wilson et al. 2004). At the top of monocyte/macrophage development, there is the lineage-determining master regulator PU.1 that, upon activation by CSF-1 (also known as M-CSF) and IL-34 (Mossadegh-Keller et al. 2013), controls the gene expression module common to all monocytes/macrophages and is the crucial regulator of myeloid lineage commitment at the fork in the road between myeloid and lymphoid fates (McKercher et al. 1996; Nerlov and Graf 1998). The transcription factor MafB represses the activity of CSF-1 (Sarrazin et al. 2009), and its expression is reduced in myeloid-committed cells in respect to HSCs. CSF-1 not only directs lineage commitment of HSCs toward CMP (common myeloid progenitors—lymphoid potential is lost) but also of GMP (granulocyte-macrophage progenitors) toward MDP (monocyte-macrophage DC progenitors—granulocyte potential is lost). Recently, the purification of the more restricted MDP population in mouse allowed to define a precursor from which monocytes and DCs are derived, that is, the distinction between CDP (common DC progenitors), a DC-restricted precursor in the BM, and cMoP (common monocyte progenitors), a monocyte-restricted BM precursor that represents the more differentiated, committed monocyte population (Hettinger et al. 2013). cMoP give rise to both monocyte subsets Ly6C^{high} and Ly6C^{low}. A cMoP monoblast type of cell has not been identified yet in humans. Figure 2.1A summarizes the sequential steps of monocyte development.

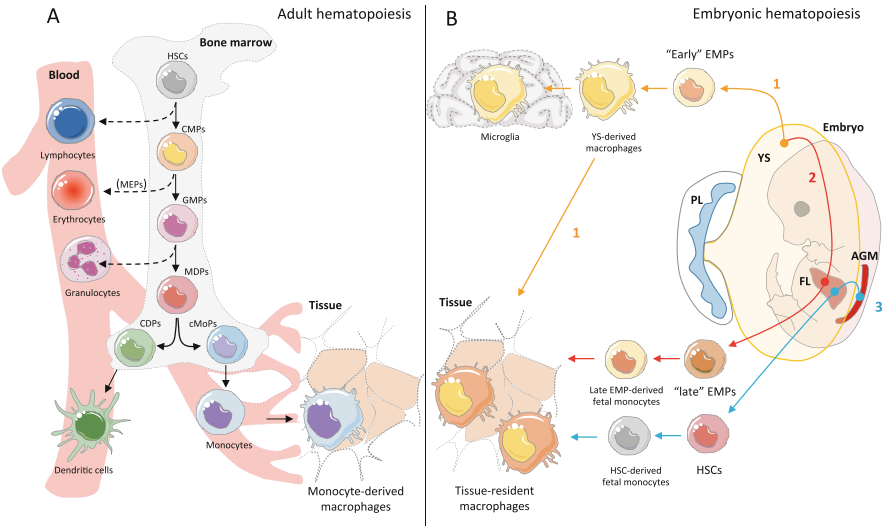

Fig. 2.1 Origin of monocyte-derived macrophages (moMϕ) and tissue-resident macrophages (trMϕ). (**A**) moMϕ are generated in the bone marrow (BM) through the tightly regulated differentiation of HSCs to progressively more committed cells up to monocytes. This hematopoiesis is defined as "definitive hematopoiesis" and occurs in fetal liver (FL) during the late phase of the embryonic development (before birth) and in BM during adulthood (after birth). Monocytes are released to the bloodstream and can be recruited into the tissue where they differentiate into macrophages. *HSCs* hematopoietic stem cells, *CMPs* common myeloid progenitors, *GMPs* granulocyte-macrophage progenitors, *MDPs* monocyte-macrophage DC progenitors, CDPs common dendritic cell progenitors, cMoPs common monocyte progenitors. (**B**) trMϕ arise from multiple sources during the embryonic development with a sequential timing: from yolk sac (YS), FL and aorta-gonads-mesonephros (AGM) regions according to three different routes. These routes are a simplification of the three main successive waves of the embryonic hematopoiesis and also of the three proposed models for macrophage ontogeny. (1) The first wave arises directly from YS, which produces "early" erythro-myeloid progenitors (EMPs) ("primitive hematopoiesis") from which YS macrophages are generated. To date, the progenitors giving rise to macrophages are poorly characterized. The first model proposes that these YS-derived macrophages represent the main precursors for the most trMϕ and not exclusively for microglia. (2) The second wave generates "late" EMPs that could migrate from YS into FL and could represent transient definitive progenitors. The second model proposes that these late EMPs represent the main precursors for most trMϕ, with the exception of microglia, through a monocytic intermediate (late EMP-derived fetal monocytes). (3) The third wave starts with the generation of immature HSCs in the AGM that colonize the FL where they establish a "definitive hematopoiesis" and maybe seed the fetal bone marrow (BM) generating HSC-derived fetal monocytes. Then, these cells will finally lead to generation of HSCs in the BM during adulthood. The third model hypothesizes that trMϕ (except microglia) arise from HSC-derived fetal monocytes, and these cells, rather than late EMPs, might generate FL monocytes. *PL* placenta

2.3.2 trMϕ Ontogeny: Primitive/Fetal Hematopoiesis

Until few years ago, it was believed that tissue macrophages derive entirely from circulating blood monocytes, through adult hematopoiesis. Given that there are

some tissues which require blood-borne precursors to replenish the pool of resident macrophages, such as the dermis (Tamoutounour et al. 2013), gut (Bain et al. 2014), mammary gland (Franklin et al. 2014), and heart (Epelman et al. 2014a, b), the resident macrophage pool of most tissues derives from embryonic precursors that colonize these tissues prior to birth and is maintained locally through in situ proliferation in adulthood (Gentek et al. 2014; Sieweke and Allen 2013). In the early 2000s, a series of elegant fate-mapping experiments, experiments in parabiotic mice, and genetically engineered mouse models (Tamoutounour et al. 2013; Yona et al. 2013; Bain et al. 2014; Epelman et al. 2014a, b; Ajami et al. 2007; Ginhoux et al. 2010; Guilliams et al. 2013; Hashimoto et al. 2013) demonstrated once and for all that trMϕ (e.g., microglia, LCs, alveolar macrophages, Kupffer cells) originate from early embryonic precursors prior to birth and that the extent to which they can originate from adult HSCs depends on the context and tissue (Ginhoux and Jung 2014). Assuming that trMϕ have an embryonic origin, the next step was to understand the embryonal hematopoiesis and from which precursor tissue macrophages derive. Mammalian embryonic hematopoiesis is a complex process that makes particularly challenging the goal of determining the exact ontogeny of fetal macrophages (for review, see Hoeffel and Ginhoux 2015). In the mouse, embryonic hematopoiesis is characterized by distinct waves, occurring in different districts of the embryo and in a sequential way. The first wave arises from the blood island of YS around E7–7.5 (embryonic day) and gives rise to the so-called erythro-myeloid progenitors (EMPs). This phase is termed "primitive hematopoiesis" and generates macrophages without going through a monocytic progenitor (*myb*-independent hematopoiesis) (Gomez Perdiguero and Geissmann 2013). Actually, EMPs have been renamed "early EMPs," to distinguish them from the "late EMPs" that arise from the YS hemogenic endothelium at E8–8.5. This phase represents the second wave of hematopoiesis and is characterized by the emergence of lympho-myeloid progenitors (Li et al. 2014). This wave is called "transient hematopoiesis" because it does not persist upon transplant in immune-compromised mice (Hoeffel and Ginhoux 2015; Hoeffel et al. 2015). At E8.5 the blood circulation is established, and EMPs are able to seed the FL, where they expand and generate fetal monocytes. Concomitantly with "late EMPs" at E8.5, at third wave arises from the intraembryonic hemogenic endothelium, which generates immature HSCs in the para-aortic splanchnopleura (P-Sh) region and proceeds to give rise to fetal HSCs in the aorta, gonads, and mesonephros (AGM) region at E10.5. Then, these precursors colonize both the FL, where they establish a "definitive hematopoiesis" (*myb*-dependent) (Hoeffel et al. 2015), and fetal BM, where they finally will generate adult BM HSCs. From E12.5 onward, the FL becomes the main hematopoietic organ within the embryo. While the embryonic origin of certain tissue macrophages is now accepted, the exact identity of progenitors, the exact pathway of differentiation to mature cells, and the transcription factors involved are still unknown. Three different models of the macrophage embryonic ontogeny have been proposed: (1) YS-derived macrophages represent the main precursors for most trMϕ (Gomez Perdiguero et al. 2015a), (2) "late EMPs" could represent the main precursors for most trMϕ through a monocytic intermediate

(Hoeffel et al. 2015), (3) fetal HSCs are the main precursors of FL monocytes, and adult macrophages (with the exception of microglia and partially LCs) arise from these definitive fetal HSCs (Sheng et al. 2015a). Which of the three hypotheses is valid remains still a matter of heated and constructive debate (Sheng et al. 2015b; Gomez Perdiguero et al. 2015b). For an exhaustive description of the three models and for an overview of the experiments on the ontogeny of macrophages, we refer the reader to the recent wonderful review by Ginhoux and Guilliams (Ginhoux and Guilliams 2016). Figure 2.1B summarizes the sequential steps of macrophage development.

2.4 trMϕ Versus moMϕ

According to the above evidence, we will consider trMϕ predominantly as macrophages derived from embryonic hematopoiesis that have colonized different tissues before birth and moMϕ as the mononuclear phagocyte population originating through definitive hematopoiesis after birth. This different origin has contributed not only to a primary distinction in terms of ontogeny but also to a secondary one in terms of functions. Generally, a conventional view implies that at steady-state the embryonic-derived trMϕ serve to maintain tissue homeostasis via surveillance of local tissue microenvironment. On the other hand, during inflammation (due to infection, disease, or trauma), it is generally accepted that circulating monocytes are predominantly recruited in the tissue, where they mediate various effector functions and differentiate into macrophages (moMϕ). To date it is not fully understood how much these two populations differ from each other and in their contribution to the initiation and resolution of the inflammatory response. Likewise, it is not known how much their functions differ due to diverse development origin ("nature") or how much overlap due to influence of the environmental cues ("nurture"). The next sections will address these issues.

2.4.1 In Situ Macrophage Expansion/Proliferation Versus Monocyte Recruitment

In healthy tissues and in steady-state conditions, trMϕ are maintained by self-renewal with a variable contribution by circulating monocytes (Yona et al. 2013; Hashimoto et al. 2013; Gomez Perdiguero et al. 2015a, b). It has been observed that in situ proliferation versus peripheral BM-derived monocyte recruitment for the maintenance of the tissue macrophages can change based on the tissue. Thus, on the one hand, we observed that the brain microglia have the potential for efficient self-renewal without the contribution of BM-derived precursors (Bruttger et al. 2015), and the same happens for resident macrophages in the liver, epidermis, lung,

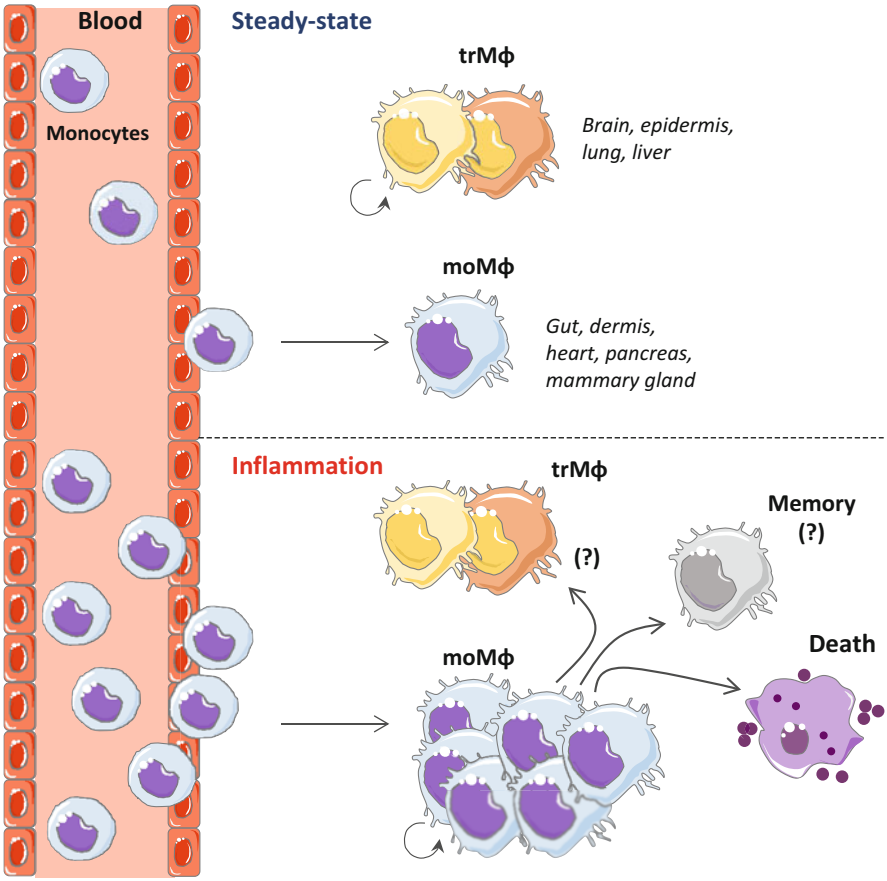

Fig. 2.2 Fate of monocyte-derived macrophages (moMϕ) in steady state and inflammation. In the steady state, tissue-resident macrophages (trMϕ) derived from embryonic progenitors populate various tissues (i.e., the brain, epidermis, lung, liver) and maintain their mass by self-renewing proliferation. In the tissues with high rate of cell turnover (i.e., the gut, dermis, heart, pancreas, mammary glands), trMϕ can originate from bone marrow (BM)-derived circulating monocytes. During inflammation, a large number of BM-derived circulating monocytes enter all tissues where they differentiate into macrophage-like cells (moMϕ), which might proliferate. The main fates of moMϕ are highlighted: (1) they die during the effector phase of the inflammatory reaction, (2) they become part of the trMϕ, and (3) they preserve the "memory" of the inflammatory challenge

pancreas, and splenic red pulp (Hashimoto et al. 2013; Chorro et al. 2009; Calderon et al. 2015). On the other hand, a crucial role for adult monocytes in replenishing the resident macrophage pool has been identified in the gut (Bain et al. 2014), dermis (Jenkins et al. 2011), heart (Epelman et al. 2014a, b; Molawi et al. 2014), and recently in the peritoneum (Bain et al. 2016).

During inflammatory conditions (upon infection, damage, or pathology), a large influx of BM-derived monocytes occurs, which differentiate into macrophage-like cells (moMϕ), and this differentiation occurs in parallel with the expansion of trMϕ

within the tissue. The fate of these moMϕ is not known. For instance, it is not clear if they mostly die (as it has been postulated) or if some of them survive becoming memory macrophages (Italiani and Boraschi 2014) (see Fig. 2.2).

Currently, the most relevant question is whether moMϕ are able to do self-maintenance in the tissue, i.e., the extent by which they survive and populate the inflamed tissue after recruitment, thereby contributing to replenishing trMϕ after the resolution of inflammation. Recent studies revealed that BM-derived monocytes display limited expression of proliferation genes as compared to FL monocytes and YS macrophages (Hoeffel et al. 2015; van de Laar et al. 2016) and often fail to stably persist in a tissue once inflammation resolves (Ajami et al. 2007; Hashimoto et al. 2013). Thus, they can be considered as "passenger myeloid cells" with respect to "resident counterparts of embryonic origin" (Gomez Perdiguero and Geissmann 2016). However, new evidence, obtained by using neonatal Csf2rb$^{-/-}$ mice with empty alveolar macrophage niche and a model of diphtheria toxin-mediated depletion of liver-resident Kupffer cells, demonstrates that the BM origin per se does not preclude the development of self-maintaining trMϕ (van de Laar et al. 2016, Scott et al. 2016).

Conversely, in the lung and brain, BM-derived monocytes do not seem to substantially contribute to the resident macrophage population after the resolution of infection or injury (Hashimoto et al. 2013; Ajami et al. 2011). In the epidermis, a small subset of moMϕ has been observed that may be remnant of monocytes recruited upon tissue damage (Sere et al. 2012), despite LCs also demonstrate a local proliferation (Chorro et al. 2009). In the liver, the nature of the damage determines the contribution of moMϕ to the pool of Kupffer cell. Upon bacterial infection, moMϕ repopulate trMϕ (Blériot et al. 2015), whereas upon paracetamol-induced injury, Kupffer cells proliferate and expand in the liver (Zigmond et al. 2014). Upon viral infection, Kupffer cells have a beneficial antiviral effect in the early phase after infection but seem to suppress the antiviral immunity during chronic infection. Moreover, it is difficult to distinguish the contribution of Kupffer cells versus infiltrating monocytes/macrophages because of the lack of distinctive phenotypical markers (Ju and Tacke 2016). Likewise, local proliferation of the resident macrophage population has been observed in atopic dermatitis (Chorro et al. 2009), while a contribution from moMϕ has been observed in ultraviolet irradiation-induced skin damage (Ginhoux et al. 2006). It has been observed that not only trMϕ but also BM-derived moMϕ proliferate in the zymosan-induced inflammatory reaction in the mouse peritoneal cavity (Davies et al. 2013). This proliferation depends on CSF-1 but is independent of IL-4, suggesting that the in vivo proliferation of trMϕ mediated by IL-4 upon nematode infection (Jenkins et al. 2011; Jenkins et al. 2013) may be restricted to type 2 inflammatory reactions. Recently, multiple fate-mapping approaches demonstrated that arterial macrophages arise from CX3CR1^{+} embryonic precursors and postnatally from BM-derived monocytes. Arterial macrophages are maintained by self-renewal and local proliferation, without a substantial contribution from blood monocytes in adulthood and after severe depletion during polymicrobial sepsis (Ensan et al. 2016). On the other hand, moMϕ are able to proliferate during pancreatic injury

(Van Gassen et al. 2015) and during hemolysis and erythrocyte damage in the red pulp spleen (Haldar et al. 2014). Monocytes contribute to the resident macrophage population also during inflammation due to pathological conditions such as atherosclerosis (Tacke et al. 2007) (where an important local proliferation of trMϕ is also observed - Robbins et al. 2013), cardiac inflammation (Epelman et al. 2014b), or aged heart (Molawi et al. 2014), in spinal cord injury (Shechter et al. 2009) but not in brain injury and neurodegeneration (Ajami et al. 2007).

2.4.2 *Are moMϕ and trMϕ Phenotypically Distinguishable?*

Upon inflammation, monocytes may infiltrate the tissue and differentiate into macrophages (moMϕ). This raises the crucial questions how to distinguish them from trMϕ, how much they are similar or differ in terms of gene expression/phenotype and in terms of their function in the inflammatory reaction, and the role of the environment in modulating their functional programming. These aspects are still not fully established. Using a genotoxic irradiation model, in which embryonic (host)- and postnatal (donor)-derived macrophages coexist in the tissue, and comparing the transcriptome between embryonic macrophages and BM moMϕ, more than 90% identity of gene expression has been observed in the lung, peritoneal cavity, and liver (Lavin et al. 2014; Scott et al. 2016; Gibbings et al. 2015; Beattie et al. 2016), although a few phenotypic markers (such as MARCO or Tim4) have been identified that could be used to phenotypically separate the two types of macrophages (Gibbings et al. 2015; Beattie et al. 2016). These findings highlight that the environment largely dictates the transcriptional programming of macrophages. However, by using the same model of macrophages depletion coupled with genotoxic irradiation, it was shown that the monocyte-derived microglia possess more than 2000 genes differentially expressed as compared to embryonic microglia (Bruttger et al. 2015).

2.4.3 *Are moMϕ and trMϕ Functionally Interchangeable?*

We will briefly review the functional contribution of trMϕ versus moMϕ during local inflammatory events, with a focus in the brain, gut, lung, and liver (Table 2.1). For the role of resident versus incoming macrophages in heart diseases (including stoke/ischemic damage) and chronic inflammatory conditions such as rheumatoid arthritis and cancer, we refer the reader to recent excellent reviews (Udalova et al. 2016; Lahmar et al. 2016; Mirò-Mur et al. 2016).

Recent fate-mapping studies have established that microglia are of embryonic origin (Ginhoux et al. 2010), persist in the brain during adulthood and in the healthy organism, and are maintained independently on BM-derived monocytes by a limited self-renewal capacity (Ginhoux et al. 2013). However, moMϕ are

Table 2.1 The main functions of trMϕ versus moMϕ in some tissues of the body

	trMϕ	moMϕ
Brain	• Monocyte recruitment • Inflammation	• Inflammation
Gut	• Maintenance of tolerogenic microenvironment	• Inflammation • Macrophage replenishment
Lung	• Immune-suppression	• Inflammation • Macrophage replenishment
Liver	• Maintenance of tolerogenic microenvironment • Protection against infections	• Inflammation • Fibrosis

massively recruited in the brain during an inflammatory event, although they do not contribute to replenishing microglia once homeostasis is restored (Ajami et al. 2011). Recently, the advantages and disadvantages of the various microglial ablation models and the origin of the "new" repopulating microglia have been discussed and reviewed (Waisman et al. 2015). At present, it is difficult to discriminate resident microglia from infiltrating myeloid cells using currently known markers and current tools (Greter et al. 2015). However, it is evident that both microglia and moMϕ play an important role in brain pathologies, as observed in experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis (Shemer and Jung 2015; Wlodarczyk et al. 2015). While infiltrating monocytes are harmful and critical in the effector phase of the disease, are highly phagocytic and inflammatory, are associated with nodes of Ranvier, and initiate demyelination, on the other side microglia remain rather inert during the early stage of EAE development, demonstrating globally suppressed cellular metabolism, but it is able to clear debris and its activation precedes the massive monocyte infiltration (Yamasaki et al. 2014). Indeed, microglia secrete numerous chemokines believed to play a role in EAE induction as responsible of the massive monocyte recruitment (Jiang et al. 2014). Therefore, an inhibition of microglia could slow down disease progression (Shemer and Jung 2015).

In the intestine, the situation is opposite, as this tissue is practically devoid of embryonic macrophages. The gut contains the largest pool of functionally specialized macrophages in the body (Gordon et al. 2014), which are essential for the tight crosstalk with microbiota, ensuring a symbiotic relationship and tolerogenic environment. Continual exposure to environmental challenge warrants constant replenishment by blood monocytes. Experiments with fate-mapping and parabiotic mouse models have demonstrated that embryonic precursors populate the intestinal mucosa during neonatal period, but they do not persist in the intestine of adult mice, and they are constantly replaced by circulating monocytes ($Ly6C^{high}$ in mice and $CD14^{++}$ in humans), which differentiate in situ into mature anti-inflammatory macrophages (Bain et al. 2014), favoring the constant need for epithelial renewal (and tissue remodeling). For this reason, in the gut, the distinction is not between trMϕ and moMϕ but rather between moMϕ (which are the resident tissue macrophages) and newly recruited monocytes. Under steady-state conditions, monocytes recruited from the blood differentiate locally into anti-inflammatory moMϕ. They

are positioned immediately beneath the epithelial barrier, where they contribute to its integrity. These macrophages are able to survey the tissue sensing and sampling the luminal content by extending processes between epithelial cells, and they produce IL-10 that facilitates the expansion of regulatory T cells. In the mouse, Ly6C^{high} monocytes are recruited during inflammation and mount an inflammatory reaction, while the resident moMϕ retain their anti-inflammatory signature. All these events are described in detail elsewhere (Gordon et al. 2014).

The role of macrophages is also essential in the lung, constantly exposed to airborne irritants and microbes. About 90% of the pulmonary macrophage population is represented by alveolar macrophages, located in the alveolar spaces. Alveolar macrophages originate from fetal liver monocytes (Thomas et al. 1976), and in steady state, they are sustained by self-renewal through local proliferation (Tarling et al. 1987). In inflammatory conditions, the repopulation of alveolar macrophages is context specific. In fact, it has been observed that during lethal irradiation, they are replenished by BM monocytes (Duan et al. 2012), while upon inoculation with influenza virus, they are replenished by self-renewal proliferation (Hashimoto et al. 2013) and, upon LPS stimulation, by both incoming monocytes and self-renewal (Upham et al. 1995). Alveolar macrophages seem to have an immunosuppressive function, as they can suppress antigen-induced cell proliferation (Holt et al. 1993) and downregulate antigen presentation by lung DCs (Careau and Bissonnette 2004). Also, alveolar macrophages seem to be protective against airway hyperresponsiveness (Guilliams et al. 2013), and, although exhibiting microbicidal and tumoricidal activities, they are less responsive than macrophages resident in other lung compartments (Hoidal et al. 1981). In the case of inflammatory diseases, such as COPD, monocytes are recruited in the lung, but their contribution to the alveolar macrophage pool remains to be determined (Vlahos and Bozinovski 2014). Using hyperreactivity mouse models with house dust mite and OVA, it has been demonstrated that alveolar macrophages dampen, whereas circulating monocytes promote, early events in allergic lung inflammation (Zaslona et al. 2014).

The liver trMϕ, Kupffer cells, represent the hematopoietic cell population among non-parenchymal cells within the liver. They arise from YS during fetal development (Schulz et al. 2012) and self-renew their population number at steady state throughout adult life with minimal contribution of blood monocytes (Hashimoto et al. 2013). Kupffer cells mainly support the tolerogenic milieu within the liver (Thomson and Knolle 2010), but their presence ensures the protection of the liver during infections (Lee et al. 2010). Recently, it has been observed that Kupffer cell death is a key signal orchestrating type 1 microbicidal inflammation and type 2-mediated liver repair upon infection (Blériot et al. 2015). Indeed, infection by *Listeria monocytogenes* induces the early necroptotic death of Kupffer cells, which is followed by monocyte recruitment and an antibacterial type 1 inflammatory response. Kupffer cell death also triggers a type 2 response that involves the hepatocyte-derived alarmin IL-33 and IL-4. This leads to the alternative activation of the moMϕ recruited to the liver, which replace ablated Kupffer cells and restore liver homeostasis (Blériot et al. 2015). Both Kupffer cells and moMϕ are involved in liver fibrosis, a common endpoint of many chronic liver diseases such as viral

hepatitis, primary biliary cirrhosis, alcoholic and NASH, or autoimmune liver disorder (Eckert et al. 2015). Generally, Kupffer cells are involved in the initiation and moMϕ in the progression phase of the fibrosis through the production of inflammatory cytokines. During disease progression, Ly6C^{hi} cells seem to develop into Ly6C^{lo} restorative macrophages, able to express MMPs and phagocytosis-related genes. These cells, if the harmful agent is eliminated, lead to resolution and can restore normal tissue architecture (Eckert et al. 2015). Otherwise, with the persistence of the initiating agent, they are responsible of anomalous repairing activity, thereby inducing fibrosis.

2.5 Conclusions

The current knowledge on the origin and role of trMϕ suggests that these cells, either coming from YS/FL precursors before birth or from blood monocytes in adulthood, have a central role in defining and maintaining tissue architecture, function, and homeostasis. The specific role of these cells obviously changes from tissue to tissue, as it is shaped (by the tissue microenvironment) to support the specific tissue requirements. It appears that the origin of these cells makes little difference in the eventual role they have within a tissue, as this is dictated by the tissue itself. Likewise, it is in most cases difficult to distinguish phenotypically, within the trMϕ, between moMϕ and YS-derived cells.

In inflammatory/disease conditions, tissue macrophages mostly act as alarmins, which do not directly exert a potent reaction against the dangerous event but that recall the specialized effector cells, the monocytes, from the blood to the affected tissue. The fate of these inflammatory monocytes, once entering the tissue to eliminate the danger, is not clear. While most of them probably die during the inflammatory reaction, it is possible that some of them survive and persist in the tissue, taking part to the phase of resolution of inflammation and tissue reconstruction/remodeling. Alternatively, or in parallel, it is possible that moMϕ, which enter the inflamed tissue from blood in successive waves, may become highly inflammatory effector cells in the initial phases of inflammation, and "healing" cells in the final phases, being differently polarized by the different tissue microenvironmental conditions. Eventually, it is possible that some of these moMϕ become part of the tissue-resident macrophage pool and develop the capacity of self-renewal. If these macrophages, should they really exist, preserve the "memory" of the past experience, or how this memory influences their response to subsequent dangerous events, i.e., how memory can influence macrophage polarization, is one of the most exciting questions in macrophage biology.

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Chapter 3
Hofbauer Cells: Placental Macrophages of Fetal Origin

Leticia Reyes, Bryce Wolfe, and Thaddeus Golos

Abstract Pregnancy complications such as preterm birth, miscarriage, maternal and/or neonatal morbidities, and mortality can be manifestations of underlying placental pathology. Hofbauer cells refer to a heterogeneous population of fetal macrophages that reside within the functional unit of the placenta known as the chorionic villus. Hofbauer cells can be detected within the connective tissue matrix of the placenta as early as 4 weeks post-conception and are present throughout pregnancy. These cells are implicated in a wide array of functions important for a successful pregnancy including placental morphogenesis, immune regulation, control of stromal water content, and the transfer of ions and serum proteins across the maternal–fetal barrier. Derangements in Hofbauer cell homeostasis are associated with placental pathologies involving infection, inflammation, and inadequate placental development. Despite a growing body of evidence that these cells are important, our knowledge about Hofbauer cell function in both normal and dysfunctional pregnancy is rudimentary. The goal of this chapter is to provide an overview of what is known about Hofbauer cell origins and their potential roles in normal and complicated pregnancy. We also review established and emerging methodologies available for the study of Hofbauer cells during in vitro and in vivo conditions.

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3.1 Hofbauer Cell Definition and Origins

Hofbauer cells are fetal macrophages that reside within the connective tissue core of the placental villous tree (Benirschke et al. 2012; Castellucci et al. 1980, 1984; Martinoli et al. 1984). Hofbauer cells were originally referred to as macrophage-like cells that were readily identified by light microscopy because of their round to ovoid shape with abundant vacuolated cytoplasm and a small nucleus (Wynn 1967; Enders and King 1970; Wood 1980). Following the use of ultrastructural and histochemical methods, it became clear that there was a great diversity of macrophages within the villus core (Enders and King 1970; Castellucci et al. 1980; Martinoli et al. 1984). Castellucci et al. (1980) reported four types of macrophages based on whether or not these cells had lamellipodia, funnel-like structures, blebs, and microplicae. Based on this classification, two of the four macrophage types were only observed in the early stages of pregnancy, while the other two forms were found in placentas collected at all gestation stages. Histochemical studies revealed a similar pattern of heterogeneity based on the degree of expression of major histocompatibility complex (MHC) class II, complement receptors, lectins, and CD14 (a lipopolysaccharide coreceptor) (Table 3.1) (Bulmer and Johnson 1984; Bulmer et al. 1988; Goldstein et al. 1988; Sutton et al. 1983). Despite the phenotypic variability of placental macrophages, they all originate from the fetus (Kim et al. 2008, 2009). Therefore, more recent studies use the term "Hofbauer cells" to collectively refer to fetal placental macrophages within the chorionic villi (Kim et al. 2012; Tang et al. 2011; Young et al. 2015).

There are several theories regarding the ontogeny of Hofbauer cells. Current consensus is that these cells have multiple origins depending on gestational stage (Benirschke et al. 2012). During early pregnancy, Hofbauer cells may differentiate from villous mesenchymal stem cells of the stroma or monocyte progenitor cells from the hypoblast-derived yolk sac (Kim et al. 2008, 2009; Takahashi et al. 1991; Seval et al. 2007). During later stages of pregnancy, Hofbauer cells may originate from fetal hematopoietic stem cells: fetal monocytes that were recruited to the placenta (Kim et al. 2008, 2009). The diverse origin of these cells may have an impact on their roles during normal and complicated pregnancy (Mantovani et al. 2013). For instance, yolk sac-derived macrophages in adult mice are linked to tissue development and morphogenesis, whereas macrophages derived from hematopoietic stem cells may be important for adult hematopoiesis and antigen presentation (Schulz et al. 2012).

3.2 The Biological Function of Hofbauer Cells

The placenta is designed to support growth and development of the fetus. Under normal conditions, the placenta undergoes a highly regulated and coordinated process of development in order to ensure an adequate exchange of nutrients and

Table 3.1 Macrophage markers present on Human Hofbauer cells (HBC) from normal pregnancies

Marker	Phenotype	Reference
ACP	Expressed in 1st and 2nd trimester HBC	Matsubara et al. (2003b)
CCR5	Expressed in 1st and 3rd trimester HBC	Soilleux et al. (2001)
CD1	Expressed in 1st and 3rd trimester HBC	Goldstein et al. (1988)
CD4	Expressed in 1st and 3rd trimester HBC	Goldstein et al. (1988)
CD11c	Expressed in 1st and 3rd trimester HBC	Goldstein et al. (1988)
CD14	Expressed in 1st and 3rd trimester HBC	Goldstein et al. (1988), Young et al. (2015), Quicke et al. (2016)
CD16	Expressed in 1st and 3rd trimester HBC	Goldstein et al. (1988)
CD68	Expressed in 1st, 2nd, and 3rd trimester HBC Expression in 1st and 2nd trimester HBC > 3rd trimester	Ingman et al. (2010), Tang et al. (2013), Bockle et al. (2008), Vinnars et al. (2010), Tabata et al. (2016)
CD163	Expressed in 3rd trimester HBC	Bockle et al. (2008), Tang et al. (2013), Tang et al. (2011), Young et al. (2015)
CD209	Expressed in 3rd trimester HBC	Bockle et al. (2008)
CR3	Expressed in 1st and 3rd trimester HBC	Goldstein et al. (1988)
CXCR4	Expressed in 1st and 3rd trimester HBC	Soilleux et al. (2001)
G6PD	Expressed in 1st and 2nd trimester HBC	Matsubara et al. (2001)
HLA-DQ	Expressed in 3rd trimester HBC	Goldstein et al. (1988)
HLA-DP	Expression in 3rd trimester > 1st trimester HBC	Goldstein et al. (1988)
HLA-DR	Expressed in 1st and 3rd trimester HBC Expression in 3rd trimester > 1st trimester HBC Multifocal distribution, usually HBC near regions of necrosis	Goldstein et al. (1988) Bulmer et al. (1988), Sutton et al. (1983) Bulmer and Johnson (1984)
IgE receptor	Expression in 2nd trimester > 3rd trimester HBC	Sverremark Ekstrom et al. (2002)
Folate receptor-β	Expressed in 3rd trimester HBC	Tang et al. (2013)

(continued)

Table 3.1 (continued)

Marker	Phenotype	Reference
LYVE-1	Expressed in 3rd trimester HBC	Bockle et al. (2008)
IFN-α	Expressed in 1st and 3rd trimester HBC	Bulmer et al. (1990)
TLR4	Expressed in 3rd trimester HBC, ↑ in chorioamnionitis	Kumazaki et al. (2004)

Abbreviations: *ACP* acid phosphatase; *CCR5* C–C chemokine receptor type 5; *CD* cluster of differentiation; *CR3* complement receptor 3 also known as receptor complex (CD11b/CD18); *CXCR4* C–X–C chemokine receptor type 4; *G6PD* glucose-6-phosphate dehydrogenase; *HLA* human leukocyte antigen; *LYVE-1* lymphatic vessel endothelial hyaluronan receptor-1; *IFN-α* interferon-α; *TLR4* toll-like receptor 4

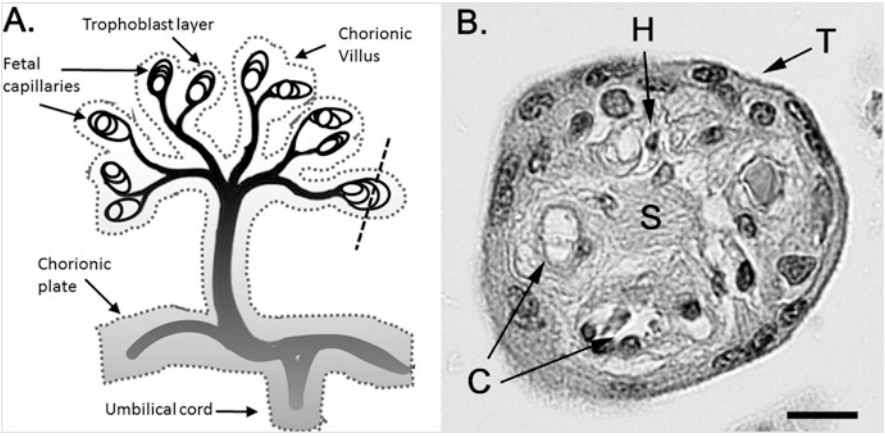

Fig. 3.1 Anatomic structure of the human chorionic villus. (**a**) A simplified diagram of the villus structure in which the vascular tree represents both the villus artery and vein. The *dashed line* indicates the position through the chorionic villus (**b**). Key: *H* Hofbauer; *T* trophoblast; *S* stroma; and *C* fetal capillaries. The *scale bar* is equal to 20 μm

waste products between the maternal and fetal circulatory systems (Kingdom et al. 2000; Gude et al. 2004). The human placenta is categorized as hemochorial: the chorion, which is the outermost fetal membrane, is in direct contact with maternal blood in the intervillous space. The main functional unit of the placenta is the chorionic villus that resembles a tree branch that is bathed in maternal blood (Fig. 3.1). The outer layer of the villus is made up of specialized epithelial cells known as trophoblasts. Within the central core of the villus are the fetal vascular network, stromal cells, and Hofbauer cells that can be found in close association with fetal capillaries and trophoblasts (Fig. 3.1) (Demir and Erbengi 1984). Hofbauer cells are present as early as 4 weeks post-conception and remain within the villous stroma through all stages of pregnancy (Castellucci et al. 1980).

Hofbauer cells typically display an M2 or M2-like phenotype (Mantovani et al. 2013). For example, DNA methylation analysis of Hofbauer cells from normal term pregnancies shows that pro-M2 genes such as CCL2, CCL13, CCL14, CD209, and A2M are hypomethylated, whereas pro-M1 genes such as TLR9, IL1B, IL12RB2, CD48, and FGR are silenced (Kim et al. 2012). Hofbauer cells constitutively express anti-inflammatory cytokines such as IL-10 and TGF-β1 (Johnson and Chakraborty 2012) and suppressors of cytokine signaling, which are negative feedback regulators of cytokine receptor signals (Satosar et al. 2004). They also produce prostaglandin E2 (Wetzka et al. 1997), which supports M2 polarization through the cAMP/CREB signaling pathway (Montero et al. 2016) and inhibits production of pro-inflammatory cytokines (Chen et al. 2012). In addition, the tissue microenvironment of the chorionic villous supports an M2 phenotype; coculture of macrophages with placental mesenchymal stem cells has been shown to induce M2 polarization in vitro (Abumaree et al. 2013). The significance of the stromal microenvironment for macrophage polarity is further underscored by the fact that Hofbauer cells can become M1 polarized when isolated from their natural environment and exposed to appropriate stimuli in vitro (Sisino et al. 2013).

M2-like characteristics of Hofbauer cells are evident by their contributions to placental angiogenesis and chorionic villus growth. In situ, Hofbauer cells typically reside in apposition to endothelial cords, blood vessels, and trophoblasts whereby they can regulate their growth or function through paracrine signaling (Cervar et al. 1999; Seval et al. 2007; Khan et al. 2000). Indeed, Hofbauer cells express high amounts of pro-angiogenic vascular endothelial growth factor (VEGF) (Cervar et al. 1999; Clark et al. 1998). They also express Sprouty (Spry) proteins Spry 1, 2, and 3, which regulate branching morphogenesis of placental villi (Anteby et al. 2005). Because of their phagocytic ability, it has been proposed that Hofbauer cells may limit placental inflammation by engulfing apoptotic bodies or necrotic cellular debris that may be recognized as danger-associated molecular patterns (Tang et al. 2013). Since Hofbauer cells reside within the stromal fluid channels of the villi, which lack a lymphatic system, it has also been suggested that Hofbauer cells may regulate stromal water content and ion transport across the maternal–fetal barrier (Demir and Erbengi 1984).

3.3 Hofbauer Cells in Obstetric Complications

There is no doubt that many obstetric complications are manifestations of underlying placental pathology or dysfunction (Altshuler and Hyde 1996; Ilekis et al. 2016). Adverse pregnancy outcomes involving preterm birth or cesarean delivery, miscarriage, maternal and/or neonatal morbidities, and mortality are sometimes associated with infectious or sterile placental inflammation or abnormal placental development. There are a limited number of reports that have examined Hofbauer cells in placentas from complicated pregnancies (Tang et al. 2013; Toti et al. 2011; Vinnars et al. 2010; Quicke et al. 2016; Tabata et al. 2016; Jurado et al. 2016;

Noronha et al. 2016). While the role of Hofbauer cells in these disease processes is yet to be elucidated, these reports provide compelling evidence that Hofbauer cells are perturbed in these pathologic conditions.

One theory that has been put forth is that Hofbauer cells may serve as a second line of defense against invading pathogens (Wood et al. 1978). However, to date there is no evidence that would support this theory. To the contrary, Hofbauer cells can be readily manipulated by invading pathogens and thus serve as reservoirs for fetal infection. Both first and third trimester Hofbauer cells have been shown to be susceptible to Zika virus infection and viral replication under in vitro conditions (Tabata et al. 2016; Quicke et al. 2016; Jurado et al. 2016). Cytomegalovirus, herpes simplex virus, and bacterial rRNA have also been detected within Hofbauer cells, which was linked to neonatal morbidity, suggesting that these cells may not be efficient at controlling infection (Satosar et al. 2004).

There may be occasions where the propensity of Hofbauer cells to be polarized toward M2 during infection may be beneficial for the fetus. This appears to be the case during HIV infection. Hofbauer cells can be infected with HIV-1, but unlike monocyte-derived macrophages, Hofbauer cells limit viral replication (Johnson and Chakraborty 2012). Constitutive expression of high levels of anti-inflammatory IL-10 and TGF-β in Hofbauer cells appears to be the mechanism for inhibiting HIV-1 replication in these cells (Johnson and Chakraborty 2012).

Disorders that induce placental inflammation can affect Hofbauer cell homeostasis. Villitis of unknown etiology (VUE) is characterized by infiltration of maternal $CD8^+$ T cells into the chorionic villi along with increased cell density of $CD14^+$ Hofbauer cells and a systemic increase in maternal and fetal chemokines (Kim et al. 2008, 2009). VUE is considered an immunologic disorder in which maternal–fetal tolerance is disrupted (Kim et al. 2009). During VUE, there is a greater density of Hofbauer cells in affected villi that also express more CD14 and are positive for Ki67 proliferation marker, suggesting that these cells are activated and proliferating (Kim et al. 2008). In VUE, Hofbauer cells along with surrounding stromal cells and endothelial cells have been shown to express greater amounts of anti-angiogenic chemokines such as CXCL9, CXCL10, CXCL11, and CXCL13 (Kim et al. 2009).

Chorioamnionitis, or inflammation of the placental membranes, is usually triggered by ascending infection from the lower genital tract (Vinnars et al. 2010). In severe cases of chorioamnionitis that involved premature labor (14–37 weeks gestation), an overall decrease in Hofbauer cell density (based on $CD68^+$ staining) was observed (Vinnars et al. 2010). However, another study that examined term placentas with chorioamnionitis reported no change in the overall density of Hofbauer cells in villi but did observe a multifocal distribution of the cells in which there were aggregates of Hofbauer cells within some villi (Toti et al. 2011).

Hofbauer cell populations from diabetic placentas show increased expression of CD68 and IL-1β, with a concomitant decrease in CD163, CD209, and IL-10, when compared to normal controls (Sisino et al. 2013). Using a streptozotocin-induced diabetes model in rats, Sisino et al. (2013) showed that hyperglycemia produced a similar profile in rat Hofbauer cells, and this was the result of activation of tissue

oxidative stress and induction of TLR-mediated signaling followed by increased production of nitric oxide (Sisino et al. 2013).

Preeclampsia is a pregnancy-specific syndrome that manifests as maternal hypertension and proteinuria. The pathogenesis of severe or early-onset preeclampsia involves poor placentation during the first trimester, with subsequent dysregulation of angiogenic factors and hypoxic–ischemic damage to the placenta (Tang et al. 2013). This disorder also features a significant reduction in the number of Hofbauer cells present in placental tissue (Tang et al. 2013). The cause for the reduction in Hofbauer cells is unknown, but due to their scavenging ability and anti-inflammatory nature, it has been postulated that their loss contributes to placental tissue damage during preeclampsia (Tang et al. 2013).

3.4 Approaches for Studying Hofbauer Cells: Strengths and Potential Pitfalls

Descriptive microscopic and immunologic studies of human placenta were the first to identify Hofbauer cells and also provide some important insights about these cells, which remain valid to this day (Wynn 1967; Enders and King 1970; Wood 1980; Castellucci et al. 1987; Castellucci et al. 1980; Martinoli et al. 1984; Fox 1967; Matsubara et al. 2001, 2003a). For example, it was established that Hofbauer cells are indeed macrophages based on their antigen expression profile, phagocytic ability, and presence of lysosomes (summarized in Table 3.1). Moreover, that Hofbauer cells are fetal rather than maternal was demonstrated by the presence of a Y chromosome in placentas from male fetuses (Kim et al. 2008, 2009). Moreover, ultrastructural morphologic and immunologic studies showed that fetal macrophages comprise a heterogeneous population (Castellucci et al. 1980; Goldstein et al. 1988), and that based on morphology, some cell types were detected throughout pregnancy, while other types were only present during early gestation (Castellucci et al. 1980).

The development of methodologies that isolate live Hofbauer cells from placental tissue created the opportunity to better define the functional capacity of these cells with in vitro culture systems (Wetzka et al. 1997; Tang et al. 2011; Khan et al. 2000; Uren and Boyle 1985). These methods utilize mechanical disruption and serial enzymatic digestion of stromal tissues followed by Percoll or Ficoll gradients (Wetzka et al. 1997; Tang et al. 2011; Khan et al. 2000; Uren and Boyle 1985). Since these cell suspensions still contain large amounts of trophoblasts and fibroblasts, antibody-based depletion by magnetic beads has been used to enrich for Hofbauer cells. One approach is to use positive selection by targeting cells that express a specific cell surface marker such as CD14 (Johnson and Chakraborty 2012). This is ideal, as long as the study acknowledges that the results of the experiment are not representative of the fetal macrophage population and that the antibody used does not inadvertently activate the targeted cell. Negative selection

methods can avoid those potential pitfalls (Wetzka et al. 1997; Tang et al. 2011), but again, results may be confounded if the heterogeneous population reflects a functional mixture of macrophages.

Modern flow cytometers allow the simultaneous measurement of several antigens on a single cell within one biological sample. The flow data generated with these systems can then be analyzed by different approaches. Multi-parameter analysis utilizes traditional flow cytometry analysis software and different gating strategies to identify multiple cell subsets within a cell type (Chattopadhyay et al. 2008). As proof of principle, we evaluated Hofbauer cell populations from normal 1st trimester macaque placentas using a panel of well-characterized markers (Breburda et al. 2006) (Fig. 3.2). By applying serial gating strategies, we identified cell subsets that may have functional significance. For example, we found that approximately 50% of Hofbauer cells identified by dual CD163+/CD64+ staining were negative for CD68, which is considered a constitutive marker for macrophages (Vinnars et al. 2010). Given that fluctuations in the number of CD68+ Hofbauer cells have been observed in various pathological states (Vinnars et al. 2010; Tang et al. 2013; Sisino et al. 2013), this marker may be a useful tool for

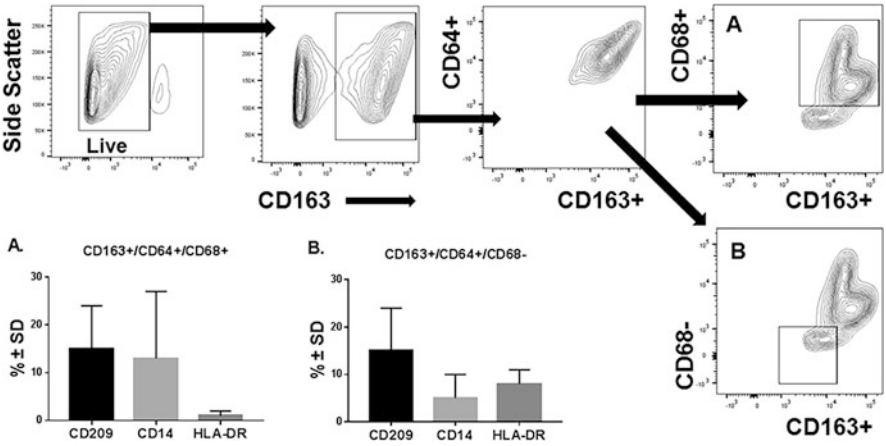

Fig. 3.2 Multi-parameter flow cytometry analysis of first trimester rhesus macaque Hofbauer cells. The *top panel* illustrates the gating strategy that was used to identify cell populations from three animals. Macaque placental tissue from gestation day 50 ± 2 days was aseptically obtained by surgery as previously described (Dambaeva et al. 2009). Decidua was removed and the villus portion of the placental was minced and treated enzymatically to disperse cells, and the cell suspension was centrifuged on a 40/20% Percoll gradient as previously described (Tang et al. 2011). The cell suspension was washed in phosphate-buffered saline before staining for flow cytometry analysis according to manufacturer instructions (bdbiosciences.com). Cells were first gated by side scatter and forward scatter and then by live/dead staining (as shown *above*). Two major Hofbauer cell subsets were identified: CD163+/CD64+/CD68+ (**a**) and CD163+/CD64+/CD68− (**b**). These subsets were further evaluated for expression of DC-SIGN (CD209), LPS coreceptor (CD14), and MHC class II (HLA-DR). Values in graphs are mean percent positive cells ± SD from 3 placental specimens

defining the biological function of these cells within the placenta. Another approach is multidimensional flow cytometry which combines all cell marker data to generate a multidimensional data space with each measured parameter being a dimension in the multidimensional space (Li et al. 2009). In this approach, cells that have similar characteristics are clustered together as a group. This approach preserves the intensity of the relationships between each of the parameters that were measured and provides a more comprehensive view of the heterogeneity of a given sample.

There is no question that evaluating human tissue has made significant advances in understanding the role of Hofbauer cells in normal and abnormal pregnancy. However, mechanistic studies into the origins and functions of human Hofbauer cells are confined to in vitro methods with cells isolated from elective terminations, miscarriages, or at delivery. Given the gestation-dependent variations in Hofbauer cell populations, cells obtained from term pregnancies cannot adequately represent what occurs at earlier gestation time points. For obvious ethical reasons, experimental manipulations are not possible in vivo, but there are animal species that also have hemochorial placentation, which have been used for placental research (Ain et al. 2006; Golos 2004; Slukvin et al. 1999; Silva and Serakides 2016).

Other species that undergo hemochorial placentation include rabbits, guinea pigs, rats, mice, bats, and nonhuman primates. These species have proven invaluable to placental research, as it is neither feasible nor ethical to conduct invasive studies on pregnant women, and in vitro models do not encompass the physiological and immunological complexity of the maternal–fetal interface. Hofbauer cells have been identified in each of these animal models. Studies of Hofbauer-like cells in the guinea pig and the little brown bat found that the Hofbauer-like cells of the bat more closely resembled human Hofbauer cells and exhibited characteristic macropinocytotic activity (Enders and King 1970). Fetal macrophages in the mouse (Takahashi et al. 1991) and macaque (King 1987) appear to share developmental and functional characteristics with human Hofbauer cells, although definitions of these cells are reliant on the markers used to identify them. Hofbauer cells are typically classified as M2 or alternatively activated cells (Brown et al. 2014; Young et al. 2015). Arginase-1 and novel mammalian lectin Ym1 may be used as markers for mouse, but not human, alternatively activated myeloid cells in the placenta. The same markers used to identify human Hofbauer cells, such as CD68, HLA-DR, and HAM56, may be used in the macaque (Table 3.1 and Fig. 3.2). While these animals have the potential to serve as models for in vivo Hofbauer cell studies, there are species-specific features in gestation length and placental structure that should be considered when using them.

A versatile yet transient organ, the placenta diverged early in its evolution and has been considered to be perhaps most variable organ among mammals. There are important physiological and anatomical differences to consider when attempting to extrapolate results from animal research to humans (Grigsby 2016; Carter 2007). Rodent and rabbit placentas, for example, have a layer of large trophoblasts in contact with the maternal decidua known as the spongiotrophoblast zone which is connected to a labyrinthine structure in which pools of maternal blood are surrounded by fetal cells (Fig. 3.3a). In contrast, primates have a branching, villous

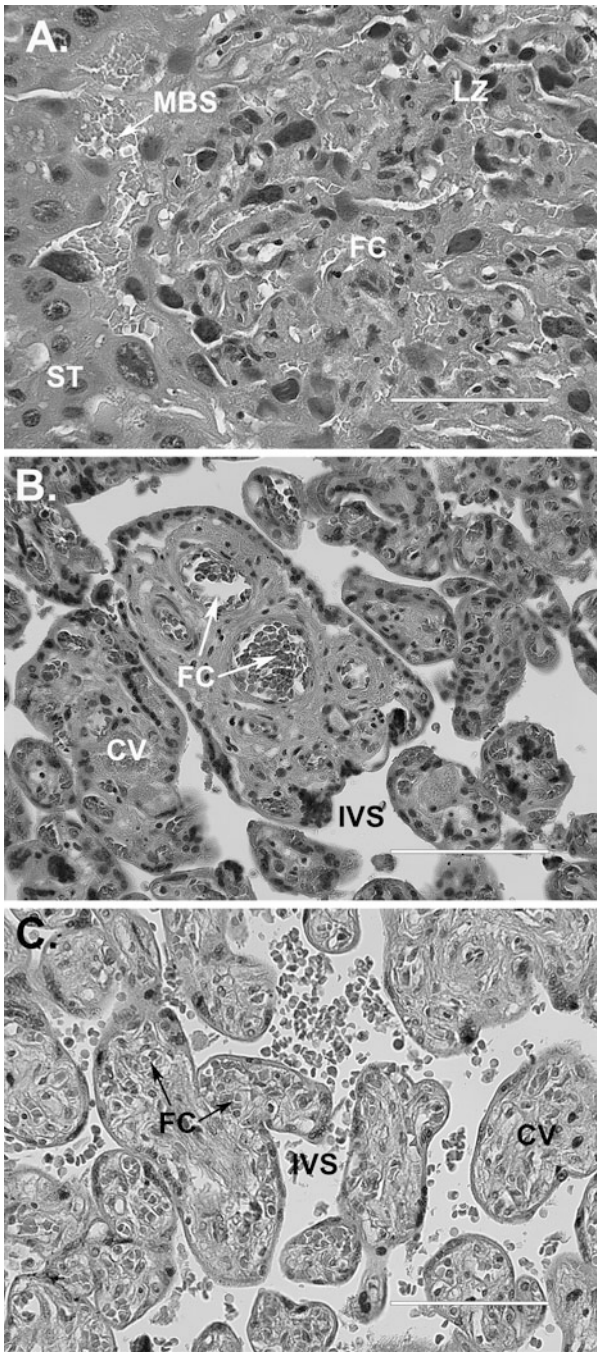

Fig. 3.3 Representative images of hematoxylin and eosin-stained hemochorial placenta in the rat—gestation day 18 (**a**), rhesus macaque—gestation day 131 (**b**), and human—term (**c**). In panel **a**: *MBS* maternal blood sinus; *ST* spongiotrophoblast layer; and *LZ* the labyrinth zone of the placenta. In panels **b** and **c**: *CV* chorionic villus, *IVS* intervillous space which holds maternal blood, and *FC* fetal capillary. *Scale bar* is equal to 100 μm

structure that is bathed in maternal blood (Fig. 3.3b and c). There is a withdrawal of maternal serum progesterone during pregnancy in rodents that does not occur in humans, fetal cells do not invade into the myometrium, and the intervillous space is fed by only a few arteries. Guinea pigs have an endocrine profile during pregnancy which is more similar to humans than mice or rats, as well as a longer gestation of approximately 70 days. However, as mentioned above, guinea pig fetal macrophages have different characteristics from human fetal macrophages.

Nonhuman primate placentas most closely resemble those of humans. Although the degree of interstitial extravillous trophoblast invasion into the uterine wall is considered shallow, nonhuman primates share a similar villous structure and have a long gestation period (~165 days), singleton pregnancies, highly similar endocrine and immune profiles, and comparable decidual cell populations to humans (Buse et al. 2014; Dambaeva et al. 2009; Breburda et al. 2006; Slukvin et al. 2001). Additionally, the similarities in decidual and placental vasculature make the nonhuman primate model useful for assessing placental angiogenesis and hemodynamic function. In vivo imaging technologies such as MRI (Benveniste et al. 2003) and contrast-enhanced ultrasound (Keator et al. 2011) are being advanced in macaques to analyze hemodynamics and cell–cell interactions. Recent work in Japanese macaques has confirmed the safety and utility of contrast-enhanced ultrasound to quantify perfusion kinetics (Roberts et al. 2016). Even as these technologies can be applied noninvasively to monitor human pregnancy, the nonhuman primate model retains the benefit of allowing researchers to intervene experimentally at various stages of gestation. This strategy is particularly useful for elucidating the contribution of Hofbauer cells in angiogenesis and villus morphogenesis, which occurs during the first trimester of pregnancy.

3.5 Conclusions

It is now clear that derangements in placental development and function can have devastating pregnancy outcomes with lasting effects on both mother and offspring. There is emerging evidence that Hofbauer cells may serve important roles in both normal and abnormal pregnancy. In order to further our understanding of Hofbauer cell biology and their function in health and disease, it is necessary to acknowledge their heterogeneous nature. Moreover, there is a need to pursue studies that elucidate whether or not Hofbauer cell diversity reflects a division of labor among these cells during normal pregnancy or is a determinant of susceptibility to invading pathogens or metabolic disorders that affect pregnancy outcomes.

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Chapter 4
Mesenchymal Stem Cells Direct the Immunological Fate of Macrophages

Patricia Luz-Crawford, Christian Jorgensen, and Farida Djouad

Abstract Mesenchymal stem cells (MSC) are multipotent stem cells with a broad well-described immunosuppressive potential. They are able to modulate both the innate and the adaptive immune response. Particularly, MSC are able to regulate the phenotype and function of macrophages that are critical for different biological processes including wound healing, inflammation, pathogenesis of several autoimmune diseases, and tumor growth. These multifunctional roles of macrophages are due to their high plasticity, which enable them to adopt different phenotypes such as a pro-inflammatory M1 and anti-inflammatory M2 phenotype. MSC promote macrophage differentiation toward an M2-like phenotype with a high tissue remodeling potential and anti-inflammatory activity but also a pro-tumorigenic function. MSC regulatory effect on macrophages is mediated through the secretion of different immunomodulatory molecules such as PGE_2, IL1RA, and IL-6. Moreover, the presence of macrophages in damaged tissue and inflammation is essential for MSC to exert their therapeutic function. In this chapter, we discuss how the interplay between macrophages and MSC mutually modulates their phenotypes and functions, orchestrates tissue repair, and controls inflammation during autoimmunity and tumor growth.

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List of Abbreviations

Arg1	Arginase 1
ASCs	Adipose-derived mesenchymal stem cells
CIA	Collagen-induced arthritis
COX-2	Cyclooxygenase type 2
CXCR4	Chemokine receptor type 4
ET-1	Endothelin-1
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KCs	Kupffer cells
LPS	Lipopolysaccharides
M1	Macrophages type 1
M2a	Macrophages type 2A
M2b	Macrophages type 2B
M2c	Macrophages type 2C
M-CSF	Macrophage colony-stimulating factor 1
MSC	Mesenchymal stem cells
NFκβ	Nuclear factor kappa-beta
NK	Natural killer
PD-1	Pathway of cell death-1
PDL1	Pathway of cell death ligand-1
PGE2	Prostaglandin E2
RA	Rheumatoid arthritis
RANK-L	Receptor activator of NFκ-β ligand
SFD-1	Stromal cell-derived factor 1
TAMs	Tumor-associated macrophages
TGF-β1	Transforming growth factor β1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TSG-6	TNF-α-stimulated gene 6
VEGF	Vascular endothelial growth factor
Ym1	Chitinase-like 3

4.1 Introduction

Mesenchymal stem cells (MSC) are adult multipotent stromal cells widely studied for their regenerative and immunomodulatory properties (Jackson et al. 2012; Le Blanc and Ringden 2007; Djouad et al. 2006, 2009; Ruiz et al. 2016). The therapeutic effect of MSC in mouse experimental disease models has been shown to be associated with their role in tissue maintenance or regeneration, support for hematopoiesis, stimulation of angiogenesis, and modulation of the immune

response (Arminan et al. 2010; Kim et al. 2012; Singer and Caplan 2011). In an inflammatory environment, MSC are able to interact with a broad range of immune cells via the secretion of several paracrine factors such as transforming growth factor (TGF)-β1, IL-6, and PGE-2 (Djouad et al. 2007; English et al. 2009) but also through cell–cell contact via Jagged/Notch or PD-1/PD-L1 pathways (Liotta et al. 2008; Luz-Crawford et al. 2012; Cahill et al. 2015). As a consequence of this interaction, MSC interact with and inhibit the function of immune effector cells inducing regulatory cell functions (Luz-Crawford et al. 2013; Glenn and Whartenby 2014). Here, we focus on the dialogue between MSC and macrophages, which results in the generation of MSC-educated macrophages and their role in (1) tissue repair, (2) immune tolerance, and (3) tumor growth.

4.2 The Interplay Between Mesenchymal Stem Cells and Macrophages in Tissue Repair

Macrophages are major players during both inflammatory and tissue repair processes. They are one of the first immune cells to arrive to the injured site in order to avoid any microbial infection and to phagocyte the remaining debris of the injured tissue (Mantovani et al. 2013; Chazaud 2014). In particular, the inflammatory subset of macrophages referred to as M1 macrophages are the first to be found in the damaged tissue. M1 macrophages are activated by LPS and IFN-γ and express high levels of the co-stimulatory molecules, inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines such as TNF-α, and low levels of Ym1 (Novak and Koh 2013). M1 macrophages induce cell proliferation to replenish the damage area and its deficiency during the process of tissue repair (Wynn and Vannella 2016). In a second wave of macrophage recruitment, another subtype of macrophages referred to as alternatively activated M2 are dominant. Upon induction with IL-4, macrophages can adopt an M2a macrophage phenotype expressing low levels of co-stimulatory molecules together with high Ym1 and Arginase-1 (Arg-1) activities, high CD206 expression, and VEGF production. All together these factors released by M2a macrophages are known to exert wound healing/pro-fibrotic functions. M2b macrophages producing pro-inflammatory cytokines are induced by immune complexes while M2c also known as an anti-inflammatory subtype of macrophages releasing high levels of IL-10 are induced by IL-10 and TGF-β1 (Novak and Koh 2013). M2 macrophages are involved in the resolution of inflammation promoting the survival, proliferation, and differentiation of the remaining or recruited progenitor cells at the site of injury. Interestingly, MSC interact with and impact on macrophage functions. Indeed, in vitro, while MSC inhibit M1 markers such as TNF-α and iNOS, they promote the differentiation of macrophages toward M2 phenotype expressing IL-10, CD206, and Arg1 (Abumaree et al. 2013; Maggini et al. 2010). These latter results are in line with observations from an in vivo model of myocardial infarction, revealing that infiltrated macrophages in the heart of mice

treated with MSC express higher levels of Arg1 and lower levels of pro-inflammatory M1 markers compared to macrophages in the mice that did not receive MSC treatment (Dayan et al. 2011).

Moreover, it has been also demonstrated that the injection of MSC in a cardiac failure model significantly increases the local recruitment of macrophages, accelerating cardiac muscle repair (Wang et al. 2015). Additionally, macrophages are critical for MSC to exert their therapeutic function since the depletion of macrophages using the lipoclodronate solution significantly decreased the therapeutic effect of MSC on cardiac regeneration. This effect was associated with the inhibition of an enhanced angiogenesis observed after MSC transplantation and occurring in the presence of macrophages (Wang et al. 2015). In another experimental model of acute kidney ischemia (AKI), MSC injection improved regeneration of the kidney promoting the switch from inflammatory M1 macrophages into an anti-inflammatory M2 phenotype. Indeed, this switch was associated with an increase in the matrix metalloproteinase (MMP)-9 activity in ischemic kidneys, which contributed to a reduction of total collagen I, and a subsequent decrease of fibrosis (Wise et al. 2014). Finally, in an experimental model of diabetes, the administration of MSC significantly increased the recruitment of macrophages with mainly a M2 phenotype. This recruitment of macrophages depends on the CXCR4/Stromal cell-derived factor (SFD)-1 axis that promotes beta cell replication and regeneration reducing diabetes progression (Cao et al. 2014). All together these data demonstrate that macrophages play a critical role in the therapeutic effect of MSC in tissue regeneration through the capacity of MSC to stimulate the migration and recruitment of M2-like macrophages into the site of damage, promoting angiogenesis and tissue remodeling (Fig. 4.1).

4.3 The Interplay Between Mesenchymal Stem Cells and Macrophages in Immune Tolerance

Macrophages are one of the main players in early stages of inflammation by playing several functions such as antigen presentation and the secretion of pro-inflammatory factors. However, the persistence of macrophage pro-inflammatory activity was shown to be associated with the development of chronic inflammatory diseases. In contrast, tissue homeostasis depends on the capacity of macrophages to adopt different phenotypes in response to different mediators promoting macrophage reprograming from pro-inflammatory M1 into an anti-inflammatory M2. This plasticity is critical for the resolution of inflammation (Jou et al. 2013).

It has been well described that MSC are able to educate tissue-resident macrophages in order to diminish local inflammation (Eggenhofer and Hoogduijn 2012). In the first in vitro approach, Kim and collaborators demonstrated that MSC were able to educate macrophages after 3 days of coculture of activated M1-like macrophages with MSC (Kim and Hematti 2009). These “educated” macrophages

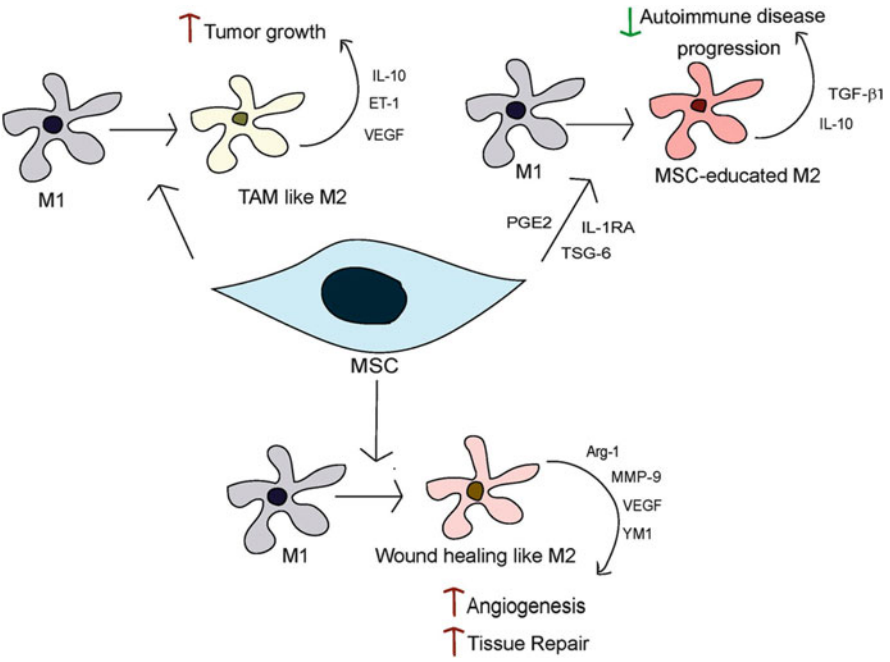

Fig. 4.1 MSC regulate macrophage immunological fate and their functions. MSC are able to induce M2-like macrophages that increase wound healing resolution. In response to inflammation, MSC induce the generation of "M2-educated macrophages" that mainly secrete IL-10 which will reduce autoimmune disease progression. In tumors, TAM macrophages will increase the survival and progression of tumor growth

produce low levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12p70 and display a higher capacity to produce IL-10 and IL-12p40 after LPS stimulation (Kim and Hematti 2009). In addition, adipose-derived MSC (ASCs) have been shown to induce M2-like macrophage phenotype independently of cell-to-cell contact when treated with either LPS or the serum of patients with acute respiratory distress syndrome (Hu et al. 2016). In another report, authors used ASC-conditioned medium to educate macrophages for chronic colitis treatment. Indeed, systemic infusion of such MSC-educated macrophages inhibited colitis in mice and reduced mortality protecting against sepsis (Anderson et al. 2013). In rheumatoid arthritis (RA), an autoimmune and inflammatory disease, macrophages are among the main players of disease progression (Udalova et al. 2016). They are significantly increased in both the synovium and the adjacent tissues (Janossy et al. 1981; Udalova et al. 2016). Indeed, macrophages in the synovial membranes of patients with RA have been described as the main initiators of T-cell infiltration and activation in an antigen-dependent manner (Janossy et al. 1981). A large body of studies has demonstrated that in RA patients, as compared to healthy individuals, there is an imbalance between pro-inflammatory M1 secreting TNF-α and anti-inflammatory M2c secreting IL-10 macrophages in favor of the first macrophage

subtype (Kennedy et al. 2011; Ye et al. 2014). Moreover, in healthy individuals there is a balance between osteoclasts and osteoblasts (bone resorption versus bone regeneration) that is completely lost in RA patients. Indeed, the production of pro-inflammatory cytokines such as IL-6 and TNF-α will stimulate the secretion of receptor activator of nuclear factor-κB ligand (RANKL) and the macrophage colony-stimulating factor 1 (M-CSF) by synovial fibroblasts, which are critical for osteoclast formation through the fusion of myeloid precursors of monocytes and macrophages (Hamilton et al. 1993; Shigeyama et al. 2000; Teitelbaum 2000). In this context, MSC have shown promising results in the treatment of arthritis. For example, ASCs significantly improve the collagen-induced arthritis (CIA) in murine model mainly through their capacity to inhibit RANK-induced osteoclastogenesis (Gonzalez et al. 2009; Garimella et al. 2015). In addition to their capacity to prevent osteoclast formation, MSC also participate in the regulation of the phenotypic switch from a pro-inflammatory M1-like to an IL-10 producing M2-like macrophage subset (Abumaree et al. 2013). In another model of liver transplantation, the MSC were able to reprogram Kupffer cells (KCs) that are resident hepatic macrophages that control innate liver immunity (You et al. 2015). Similar to typical macrophages, they can display different phenotypes, depending on the stimuli they receive, to promote hepatic immune tolerance (Movita et al. 2012). Several studies have demonstrated that the negative or positive outcome in liver injury will strictly depend on the phenotype of KCs (Movita et al. 2012; Ahsan et al. 2013; Akamatsu et al. 2003). In this context, MSC were able to induce the switch of KCs from a M1 phenotype into an M2 phenotype, which significantly contributed to liver allograft tolerance (You et al. 2015). In a sepsis experimental model, the infusion of MSC has been shown to polarize pro-inflammatory macrophages into an anti-inflammatory phenotype resulting in an improvement of survival. However, when macrophages were depleted using either the lipoclodronate or specific antibodies against IL-10, the therapeutic effect of MSC was completely lost (Nemeth et al. 2009).

The mechanism by which MSC modulate macrophage polarization is still under investigation. However, it has been demonstrated that MSC treated with TNF-α, the main pro-inflammatory cytokine produced by M1-like macrophages, significantly increase the secretion of anti-inflammatory molecules such as TNF-α-stimulated gene 6 protein (TSG-6) (Torihashi et al. 2015). Recently, it has been reported that intravenous administration of MSC prompts the generation of M2 alveolar macrophages that will induce immune tolerance (Ko et al. 2016). Moreover, prostaglandin E2 (PGE2) through the upregulation of Cox2 expression as well as other components of the arachidonic acid pathway reprograms macrophages into a M2-like phenotype (Nemeth et al. 2009). TSG-6 prevents the Toll-like receptor 2 (TLR2) signaling in macrophages via CD44, which will inhibit NF-κB and decrease macrophage inflammatory response (Choi et al. 2011), and PGE2 promotes the polarization of macrophages toward an M2-like phenotype (Uccelli and de Rosbo 2015). Others and we have demonstrated that the production of IL-1 receptor antagonist (IL-1RA) by MSC plays a critical role in the modulation of macrophage phenotype promoting their differentiation toward an M2-like phenotype (Ortiz et al. 2007;

Luz-Crawford et al. 2015). It has been proposed that in vivo the beneficial effect of MSC is initiated in the lung where MSC migrate after intravenous administration. This was associated with an enhanced polarization of macrophages toward an M2-like phenotype resulting in the increased IL-10 levels in the lung in response to MSC injection. Moreover, the protective role of MSC on hepatic injury was significantly decreased upon administration of an anti-IL10 neutralizing antibody (Lee et al. 2015). All together these data suggest that the therapeutic effect of MSC in autoimmune disorders is associated with the generation of M2-like macrophages that increase IL-10 production to dampen pathogenic inflammation.

4.4 Mesenchymal Stem Cells Promote Tumor Progression Through Macrophages

Macrophages are one of the most represented leukocyte population within solid tumors. Indeed, their role in tumor cell growth depends on the phenotype acquired by macrophages in the tumor microenvironment (Lamagna et al. 2006). Anti-tumorigenic-activated M1-like macrophages are able to stimulate activation of resting NK cells and recruitment of pro-inflammatory T cells into the tumor, while tumor-associated macrophages (TAMs) are alternatively activated M2-like macrophages that stimulate anti-inflammatory responses exerting pro-tumorigenic functions (Fig. 4.1) (Solinas et al. 2009; Wong et al. 2009). Because it has been shown that the deficiency in M1 macrophage polarization significantly increases tumor progression (Kondo et al. 2016), one of the main targets for cancer therapy is the modulation of macrophage polarization in the tumor microenvironment from a pro-tumorigenic M2-like to an anti-tumorigenic M1-like macrophage phenotype. In this context, MSC have been shown to promote tumor progression by increasing the generation of M2-like macrophages, calling into question the use of MSC to treat tumors (Jia et al. 2016). In line with this study, Yamada and collaborators have shown that MSC infusion significantly favors tumor progression by controlling macrophage differentiation and function (Yamada et al. 2016). Indeed, MSC induce generation of a particular M2-like macrophage subset able to inhibit the cytotoxic activity of both NK and $CD8^+$ T cells by reducing the expression of NKp44, CD69, and CD25 markers, and production of IFN-γ, and by inducing generation of T regulatory cells, which will lead to an improvement of tumor growth (Mathew et al. 2016). Furthermore, MSC secreting VEGF and ET-1 will significantly promote tumor progression by increasing the number of M2-like macrophages within tumors inducing a tolerogenic environment and promoting tumor angiogenesis (Yamada et al. 2016). Interestingly, the cross talk between MSC and macrophages also favors the tumor to escape from immune surveillance since M1-like macrophages enhance the capacity of MSC to promote tumor growth in vivo. Moreover, primed MSC produce significantly higher levels of iNOS and MCP1 as compared to unstimulated MSC, which increases recruitment of macrophages to the tumor sites. Furthermore,

IL-6 secreted by stimulated MSC polarizes infiltrated macrophages into an M2-like phenotype. Thus, in the presence of anti-tumorigenic M1-like macrophages in the tumor microenvironment, MSC seems to act as sensors and switchers of inflammation accelerating tumor progression (Ren et al. 2012).

In summary, cellular interactions between MSC and immune effectors, in particular macrophages, in the tumor microenvironment play a pivotal role in the establishment of tumor immune escape.

4.5 Conclusions

The dialogue between MSC and macrophages has a critical role for their phenotype and function. In the context of wound healing, MSC induce generation of an M2-like phenotype which will control the resolution of inflammation and promote angiogenesis and tissue repair. In inflammatory disease models, MSC inhibit pro-inflammatory M1-like macrophages promoting M2-like phenotype that will reduce autoimmune disease progression. However, in tumor, MSC will support the anti-inflammatory microenvironment by generating TAMs with pro-tumorigenic growth activities. In conclusion, M2-like macrophages induced by MSC improve tissue repair, inhibit inflammation, and support tumor growth. Thus, the specific mechanisms by which MSC are able to interact with macrophages have to be clearly defined to ensure safe clinical use of MSC.

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Chapter 5
Monocyte/Macrophage: NK Cell Cooperation—Old Tools for New Functions

Elżbieta Wałajtys-Rode and Jolanta M. Dzik

Abstract Monocyte/macrophage and natural killer (NK) cells are partners from a phylogenetic standpoint of innate immune system development and its evolutionary progressive interaction with adaptive immunity. The equally conservative ways of development and differentiation of both invertebrate hemocytes and vertebrate macrophages are reviewed. Evolutionary conserved molecules occurring in macrophage receptors and effectors have been inherited by vertebrates after their common ancestor with invertebrates. Cytolytic functions of mammalian NK cells, which are rooted in immune cells of invertebrates, although certain NK cell receptors (NKRs) are mammalian new events, are characterized. Broad heterogeneity of macrophage and NK cell phenotypes that depends on surrounding microenvironment conditions and expression profiles of specific receptors and activation mechanisms of both cell types are discussed. The particular tissue specificity of macrophages and NK cells, as well as their plasticity and mechanisms of their polarization to different functional subtypes have been underlined. The chapter summarized studies revealing the specific molecular mechanisms and regulation of NK cells and macrophages that enable their highly specific cross-cooperation. Attention is given to the evolving role of human monocyte/macrophage and NK cell interaction in pathogenesis of hypersensitivity reaction-based disorders, including autoimmunity, as well as in cancer surveillance and progression.

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5.1 Introduction

During the last decades, growing interest of the close interplay between the innate and adaptive immunity mechanisms brought the intensification of the studies concerning the particular role of the two main cell populations, namely, NK cells and monocyte/macrophages, that realize the innate and participate in the adaptive immunity. The matter of interest is the development of these two cell types creating the first line of protection against pathogens and their specialization during evolution of organisms, beginning from their independent activities and coming to very close cooperation in human immune system. The role of both cell types acting on the border of and integrating innate and adaptive immunity can be understood only on the basis of the new data concerning knowledge about the mechanisms of their differentiation into specific subsets under influence of environmental components including self- and non-self-effectors. The presented data confirm that immune system has to be considered as continuous spectrum of cooperating elements and that traditional approaches should be often verified and corrected.

In this chapter we describe the evolutionary development of NK cell and monocyte/macrophage populations and summarize data concerning their molecular mechanisms of the complementary as well as redundant interactions in human immune system. According to the recent investigations, these two cell types reveal many similar functions although realized by various molecular mechanisms determined by microenvironment and their direct interactions in health and disease. Majority of included data concern human immune system, until otherwise stated. For the reason of the limited volume, the relevant published reviews comprising the recognized properties and functions of NK cells and macrophages as well as their interactions are limited to certain hypersensitivity reaction-based disorders and cancer diseases. The chapter is devoted to recent studies revealing the new, specific molecular mechanisms of regulation of NK cells and macrophages that enable their new discovered functions and cross cooperation.

5.2 Origin of Macrophages and NK Cells

Cooperation between macrophages and NK cells brings about more potent, successful defense against pathogens and cancer cells. These cells are effectors of innate immunity that is indispensable for both unicellular and multicellular organisms for surviving and reproduction. Discrimination between self and non-self demands mechanisms of recognition of ligand by specific receptor, transmission of signal, and effective response. Cooperation of macrophages and NK cells in vertebrates is possible due to a long evolution of immune mechanisms and immune cells of invertebrate immune systems (Fig. 5.1).

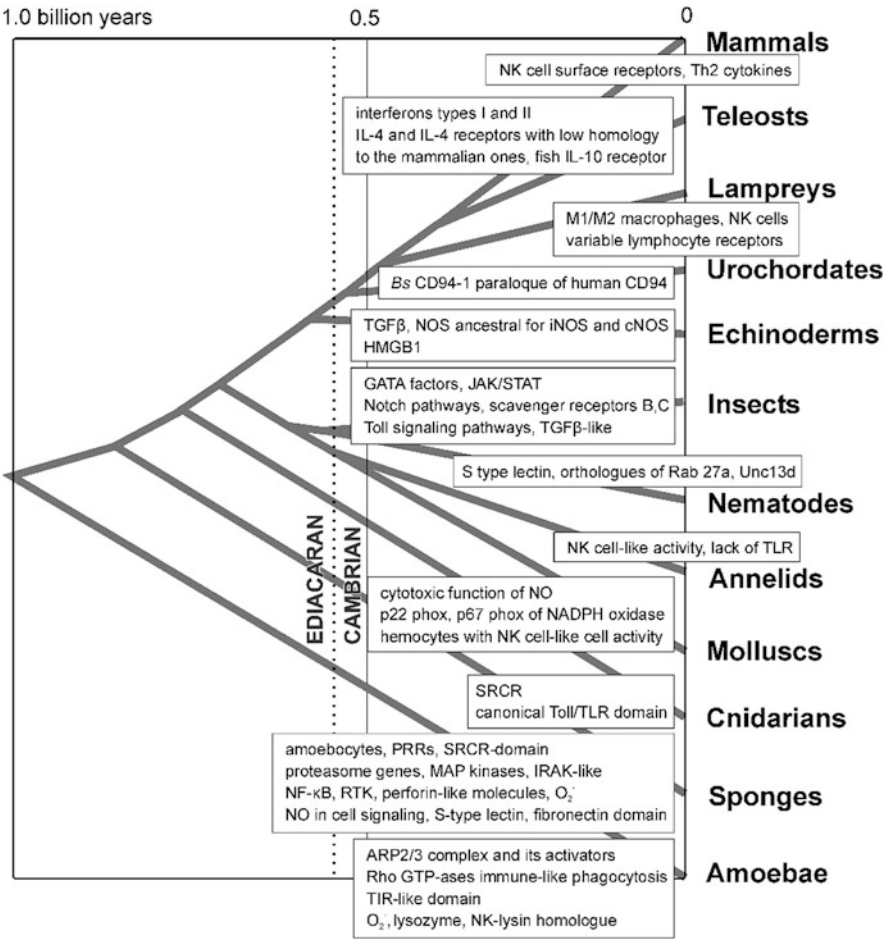

Fig. 5.1 First appearance of molecules or processes is overlaid on phylogenetic relationships among taxa referred to in the text and mentioned in Dzik (2010). Abbreviations: *ARP2/3 a*ctin-*r*elated *p*roteins 2 and 3, *HMGB1* high mobility group box 1, *NK-lysin* natural killer cell-lysin, *NOS* nitric oxide synthase (*i*nducible or *c*onstitutive isoforms), *Nox* phagocyte NADPH oxidase, *PRRs* pattern recognition receptors, *Rab27a* the small GTPase, *RAG* recombination-activating genes, *RTK* receptor tyrosine kinase, *SRCR domain* scavenger receptor cysteine-rich domain, *TIR* Toll, interleukin-1 receptor and plant disease resistance genes. Unc13d gene codes the granule priming factor Munc13-4

5.2.1 Macrophage Differentiation in Vertebrates and Invertebrates

Considerable population of cells, which are definitive macrophages, occurs in vertebrate tissues. They express high levels of F4/80 marker (dos Anjos Cassado 2017; Schulz et al. 2012). According to available evidence, most populations of

macrophages in mice derive from embryonic precursors in the yolk sac well before any monocyte or granulocyte, suggesting that they arise from some hematopoietic stem cell through a pathway that bypasses the monocytic series. They quickly mature into "fetal macrophages." From the yolk sac, these macrophages invade the mesenchyme of the head and from there other developing organs, where macrophages can be seen dividing (Cuadros et al. 1993; Sorokin et al. 1992). They disperse with the blood circulation, before the appearance of hematopoietic islands and the onset of hematopoiesis in the liver (reviewed by Lichanska and Hume (2000)). Then, with the postnatal formation of the bone, fetal liver hematopoiesis declines and is replaced by bone marrow hematopoiesis. This definitive hematopoiesis is the source of circulating monocytes (Ginhoux and Guilliams 2016).

Mice macrophages exist independently of monocytes in the steady state, or emerge during certain types of inflammation. The hematopoiesis in bone marrow is the source of circulating monocytes (Ginhoux and Guilliams 2016). A minor proportion of lymphocyte antigen 6c positive (Ly6C+) blood monocytes differentiate into blood lymphocyte antigen 6c negative ($Ly6C^{-}$) monocytes that function as vascular macrophages (Carlin et al. 2013;Yona et al. 2013). Remaining Ly6C+ blood monocytes enter tissues and become either macrophages, for example, in the gut and dermis (Bain et al. 2013; Jakubzick et al. 2013; Tamoutounour et al. 2012), or remain undifferentiated, acquire antigens, and migrate to the draining lymph nodes (Jakubzick et al. 2013). Several reports have suggested that the yolk-sac-derived cells are the exclusive progenitors of microglia, that is, the resident macrophages of the brain (Ginhoux et al. 2010; Hoeffel et al. 2012), and that they contribute significantly to macrophage population resident in the skin, spleen, pancreas, liver, and lungs (Schulz et al. 2012). In tissues such as the kidney and lungs, macrophages have a chimeric origin being derived from the yolk sac ($F4/80^{high}$) and bone marrow ($F4/80^{low}$) (Ginhoux and Guilliams 2016).

Some tissues' macrophages, including those in the bone marrow, are maintained mainly by self-renewal and longevity (Jakubzick et al. 2013; Yona et al. 2013; Schulz et al. 2012; Murphy et al. 2008; Hashimoto et al. 2013), even during inflammation (Davies et al. 2011; Jenkins et al. 2011; Hashimoto et al. 2013; Ajami et al. 2011). Macrophages of different origin may coexist, which suggest their different functions (Ginhoux and Guilliams 2016).

The molecular pathways controlling hematopoiesis in mammals are very conserved, and their roots can be traced to bony fish (teleosts). The early macrophages in the zebrafish embryo are most likely homologous to the primitive/fetal macrophages occurring in mammalian and avian embryos (Zapata et al. 2006). As a result, most (if not all) of the critical hematopoietic transcription factor genes identified in mammals have orthologues in zebrafish (Davidson and Zon 2004).

In the zebrafish embryo (Herbomel et al. 1999), macrophages originate from the ventrolateral mesoderm (early macrophages). These macrophage precursors migrate to the yolk sac and differentiate there. From the yolk sac, many invade the mesenchyme of the head, while others join the blood circulation. These macrophages are capable of phagocytosis of apoptotic corpses, as well as of engulfing

and destroying bacteria injected intravenously. In the head, macrophages spread in the whole mesenchyme and from there invade the epidermis, retina, and brain. Within few days after fertilization, all macrophages in the brain and retina transform into "early (amoeboid) microglia" (Herbomel et al. 2001). The second site of hematopoiesis is the anterior mesoderm. It remains unknown if the macrophages originating there represent a transient wave of "primitive" macrophages that are later replaced by monocyte-derived macrophages, or if they are retained into adulthood (Herbomel et al. 1999). The teleost immune system differs from that of mammals in that fish lack lymph nodes and bone marrow. The major lymphoid organs in teleosts are the thymus, anterior (head) kidney, and spleen (Pastoret et al. 1998). As in other vertebrates, all of the teleost blood lineages are believed to originate from a pool of pluripotent, self-renewing hematopoietic stem cells.

To explain evolutionary origin of vertebrate macrophages, one has to trace their homology with hemocytes of invertebrates. Knowledge of invertebrate hematopoiesis is based mainly on the *Drosophila* model. The process of blood cell formation in *Drosophila* is similar to those in vertebrates both in respect to the hemocyte formation and to signaling molecules involved into the hematopoiesis. Production of these cells occurs during all life stages of insects. The embryonic and larval phases of hematopoiesis correspond to the formation and expansion of self-renewing tissue-resident macrophages. Lymph gland hematopoiesis resembles vertebrate definitive hematopoiesis and gives raise to all differentiated *Drosophila* blood cell types. The differentiation of *Drosophila* blood cell progenitors depends on the GATA transcription factor Serpent (Srp) (Rehorn et al. 1996), in combination with the friend of GATA (FOG) transcription factor U-shaped (Ush) (Fossett et al. 2001; Waltzer et al. 2002). It is reminiscent of the role of GATA factors and GATA–FOG complexes in vertebrate hematopoiesis (Visvader et al. 1995; Tsang et al. 1997). It appears that many transcription as well as growth factors and signaling pathways involved in regulation of *Drosophila* hematopoiesis exhibit highly conserved function in vertebrates (Ref in Gold and Brückner 2014).

5.2.2 *The Evolutionary Roots of Natural Killer Cells*

Natural killer (NK) cells are large granular lymphocytes that recognize and eradicate a wide range of tumor cells as well as cells infected with certain viruses. Cells displaying cytolytic activity appeared early in evolution. In the hemolymph of *Planorbarius corneus*, (snail) two types of cells were identified: glass-adherent macrophage-like spreading hemocytes and nonadherent round hemocytes. The round hemocytes lysed the K-562 human cell line, typically used for NK cytotoxicity test (Franceschi et al. 1991). Natural cytotoxic activity, which is functionally similar to that of mammals, has been found in annelids, clams, crayfish, sea urchins, and tunicates (reviewed in Suzuki and Cooper 1995). Thus, the activity of NK-like cells is apparently dissociated from phagocytosis already at the level of invertebrates. Cytotoxic cells that are functionally similar to NK cells have been identified

in several vertebrate species (bony fishes, amphibians, reptiles, and birds) (Yoder and Litman 2011). NK-like cells, characterized by their morphology, lack of variable lymphocyte receptors (as antigen receptors), and transcriptional program, have also been identified in lampreys (Hirano et al., unpublished observations). Moreover, hemocytes with cytotoxic function and lymphocyte-like cell morphology have been described in tunicates (chordates) (Parrinello 1996) that are most closely related to vertebrates.

The cytotoxic cells release vesicles containing toxic molecules, and the process is controlled by Rab27a and Unc13d. Orthologs of Rab27a and an ancestral gene similar to Unc13d have been found in *Caenorhabditis elegans* (Fukuda et al. 2004). Unc13d localizes within cytotoxic granules at the immunologic synapse (Feldmann et al. 2003). Unc13d is essential for the priming step of secretion of cytolytic granules that precedes vesicle membrane fusion. Rab27a is the small GTPase involved in exocytic processes. Cytotoxic molecules, perforins and granzymes, are known from invertebrates (McCormack and Podack 2015). It is clear that in lower invertebrates the cytolytic function of NK cells is accomplished by NK-like cells. Recognition of NK cell targets depends on cell surface receptors. When we look at the phylogenesis of receptors on NK cells, there are only loose relationships between invertebrates and vertebrates as well as between different vertebrates.

Analysis of the gene expression during allorecognition in *Botryllus schlosseri* (Urochordata, the invertebrate chordates that are most closely related to vertebrates), documented a gene (named BsCD94-1), differentially regulated during allorecognition, coding for a type II transmembrane protein with a C-type lectin-binding domain (Khalturin et al. 2003). The corresponding receptor is expressed on cell surfaces of subpopulation of blood cells. BsCD94-1 is similar to vertebrate CD94 and NKRP1. CD94 in vertebrates is one of the markers for natural killer cells and binds to MHC class I molecules. The gene encoding protein related to the CD94/NK cell group 2 (NKG2) subfamily of the killer cell C-type lectin receptors was identified in teleostean species (Sato et al. 2003). However, analysis of gene sequences proves that the fish and mammalian gene clusters of NK cell receptors (NKRs) arose independently by duplications from different ancestral genes (Sato et al. 2003). This suggests that all of the phylogenetic relationships between the fish and human genes in these two clusters are of the paralogous rather than orthologous type. Similarly, the urochordate receptor identified in *B. schlosseri* (Khalturin et al. 2003) is not orthologous to the human CD94 protein, although it is a group V C-type lectin (Sato et al. 2003).

Some structural similarities exist between bony fish receptors, such as NITR ("novel immune-type receptor") family (Yoder 2004), and mammalian receptors of the "leukocyte immunoglobulin-like receptor" (LILR) and "killer cell immunoglobulin-like receptor" (KIR) families (Yoder and Litman 2011). These findings place NKR and receptors on B and T cells at the same stage of evolution. One can conclude that only the cytolytic function of mammalian NK cell is ancestral, and this function is shared with other cytolytic lymphocytes. Heterogeneity among receptor genes is seen even in the same group of mammals. Certain single-copy NKR genes that are present in one order of mammals are present as

expanded multigene families in other mammals, as was found in rodents. This phenomenon possibly results from the influence of pathogens, mainly viruses, on the evolution of NKR (Hao et al. 2006).

5.3 Phagocytosis

Basically, blood cells of all coelomate animals exhibit a division of work, including ability to phagocytosis which appeared very early in the evolution. Sponges, the phylogenetically oldest metazoa, use both oxidative (Peskin et al. 1998) and enzymatic mechanisms (Mukherjee et al. 2016) to ingest phagocytized bacteria on which they feed. Many lysosomal enzymes such as phosphatases (Bhunia et al. 2016) and proteases have been recognized in sponges (Krasko et al. 1997). Phagocytosis is also the main system of sponge defense. The demosponge *Suberites domuncula* recognizes Gram-negative bacteria; the ligand LPS (an endotoxic component of the outer membrane of Gram-negative bacteria) is recognized by the pattern recognition receptor (LPS-interacting protein), which interacts with a cytosolic adapter, recruit myeloid differentiation primary response protein 88 (MyD88)-like protein (Wiens et al. 2005). MyD88 functions as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways. As a result of LPS binding, perforin-like molecule is upregulated (Wiens et al. 2005). In addition, the receptor-mediated defense systems against Gram-positive bacteria (peptidoglycan-mediated endocytosis and lysozyme as effector) (Thakur et al. 2005) and fungi (the (1→3)-β-d-glucan-binding protein receptor and fibrinogen as effector) (Perović-Ottstadt et al. 2004) exist in sponge as well. These evolutionary oldest metazoans are provided with the three key defense arrays of the innate immune system, required for a successful survival in an environment highly loaded with microbial threats.

The sponge perforins show considerable sequence similarity to the mammalian macrophage-expressed protein (Wiens et al. 2005). One of the oldest membrane attack complexes of complement is the perforin-2/macrophage-expressed protein 1, and it is known that small granules in NK cells also contain aside of proteases (granzymes) the perforin. In sponges, only perforin-2 was present; then, additional proteins in the membrane attack complex (MAC) of complement and perforin-1 of NK cells and CTL evolved (McCormack and Podack 2015). It is reasonable to think that perforin-1 likely originates from perforin-2 to eradicate virally infected or transformed host own cells by NK cells or CTL (D'Angelo et al. 2012).

Phagocytosis is initiated by the adherence of the antigen to the macrophage cell membrane. Adherence induces membrane protrusions called pseudopodia to extend around the attached material. Fusion of pseudopodia encloses the material within a membrane-bound structure called a phagosome. A phagosome moves toward the cell interior where it fuses with a lysosome to form a phagolysosome. The fusion results in the release of acidic and enzymatic lysosomal contents into the phagosome and subsequent degradation of phagolysosome contents (Jutras and

Desjardins 2005). Phagocytosis is only one of the diverse actin-dependent processes that occur in all eukaryotic cells. Initial receptor interaction with its ligands leads to actin polymerization and internalization (Aderem and Underhill 1999). Actin polymerization requires one or more of the following assembly factors: (i) the *a*ctin-*r*elated *p*rotein 2 and 3 (Arp2/Arp3) complex that produces actin network with the help of activating proteins, (ii) formins that produce linear filaments, and (iii) spire that also produces linear filaments (Chhabra and Higgs 2007). Despite the high variability of phagocytosis mechanisms, they share the same mechanism of actin polymerization that requires the contribution of the Arp2/3 complex and Rho GTPases as key regulators (Kinchen and Ravichandran 2008). As phagocytosis depends on cytoskeleton remodeling, the actin, tubulin, non-muscle myosin, and several actin-binding proteins are among the conserved phagosomal proteins. Among signaling molecules several GTPases and their effectors are conserved as well. The small GTPases are mostly represented by the Rab family members that are involved in vesicular fusion and trafficking (Yutin et al. 2009).

Several proteins characteristic for the endoplasmic reticulum (ER) were found among other conserved proteins, proving the apparent role of ER in phagosome formation and/or maturation (Yutin et al. 2009).

Phagocytosis and killing of microbes are triggered by innate immune recognition receptors, which activate nuclear factor NF-kB, type I interferon (IFN), or other signaling pathways of inflammasome, which, in turn, induce the production of a variety of pro-inflammatory and antiviral cytokines and chemokines, resulting in initiation of adaptive immune response (Iwasaki and Medzhitov 2004). Vertebrate macrophages are activated by non-self-agents and are able to respond through the release of biologically active molecules acting in a defense. In vertebrates, germline-encoded pattern recognition receptors (PRRs), including scavenger receptors, C-type lectin receptors (CLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and other DNA sensors, are responsible for the initiation and regulation of innate immune responses (Akira et al. 2006; Takeuchi and Akira 2010; Paludan and Bowie 2013).

5.3.1 Receptors Used in Phagocytosis

Phagocytosis involves variety of receptors, and ingestion of bacteria is frequently the overall result of internalization via several receptors (Underhill and Ozinsky 2002). For example, at the cell surface, a Gram-positive bacterium is likely to be recognized by TLR2 (lipoproteins), but after phagocytosis, it is likely to be recognized also by TLR9 (bacterial DNA). Similarly, a particular Gram-negative bacterium will be recognized by TLR4 (LPS) but may also be flagellated and therefore recognized by TLR5 as well. Thus, the net inflammatory response of a macrophage or dendritic cell to TLR stimulation may be defined not only by which TLR is activated but also by which combination of TLRs is activated (Underhill 2007). Scavenger receptors are pattern recognition receptors (PRRs), which activate

phagocytosis, innate immune response, and inflammation. They contain cysteine-rich domain, which is an ancient and highly conserved protein module (Pancer 2000). This module has already been found in sponge receptors (Blumbach et al. 1998). The cysteine-rich domains are characteristic for type I macrophage scavenger receptor (SRCR), human CD6 antigen (group B scavenger receptor), and macrophage M130 antigen (also referred to as CD163). The expression pattern of M130 implies its functional role in the anti-inflammatory response of monocytes (Buechler et al. 2000). Various classes (A–H) of scavenger receptors are distinguished depending on which kind fo polyaninic ligands such as bacteria, apoptic host cells, modified lipoproteins they bind. Genes coding for class C receptors are known from *Drosophila*, amphioxus, and lamprey (ref. in Dzik 2010).

Phagocytosis is not only a tool to combat microorganisms but is crucial for tissue homeostasis and remodeling (Liao 2005; Nakanishi et al. 2011). During development of organisms, embryonic cells still undergo apoptosis. These cells are selectively and rapidly eliminated by phagocytosis, for instance, in *Drosophila* by macrophage-like hemocytes or glia (Abrams et al. 1993; Sonnenfeld and Jacobs 1995). The cellular machinery promoting phagocytosis of corpses of apoptotic cells is well conserved from worms to mammals (Ziegenfuss et al. 2008). Among scavenger receptors involved in phagocytosis is a Croquemort (Crq). It is a homologue of CD36 (Silverstein and Febbraio 2009). Crq belongs to a class B scavenger receptor family, recognizes apoptotic cells, and is specifically expressed in hemocytes/macrophages of *Drosophila melanogaster* (Franc et al. 1996). Human CD36 acts as a scavenger and also binds apoptotic cells in combination with the macrophage vitronectin receptor and thrombospondin (Savill et al. 1992). The Nimrod proteins form a superfamily that also includes proteins that are encoded in the human genome (Kurucz et al. 2007). These proteins are related to eater and draper from *Drosophila* and CED-1 from *C. elegans*. Draper is a paralogue of LPS recognition protein (Awasaki et al. 2006). Eater is an EGF-like repeat transmembrane receptor on hemocytes (Kurucz et al. 2007). It is required for efficient phagocytosis of Gram-positive bacteria and enabling hemocyte attachment at the sessile compartment (Kocks et al. 2005). CED-1 from *C. elegans* was identified as an ortholog of human SREC-I (Suzuki and Nakayama 2007).

In the bony fishes, scavenger receptors are expressed on population of nonspecific cytotoxic cells, corresponding to NK cells (Kaur et al. 2003) and on phagocytic cells. In mammals, scavenger receptors SRAI/II; the SR A-type macrophage receptor with collagenous structure (MARCO); SR BIII (CD68); CD36, an SR B-like receptor; and lectin-like oxidized low-density lipoprotein receptor (LOX-1) (Peiser et al. 2002) mediate recognition and uptake of bacteria. MARCO is a particularly important receptor for the ingestion of unopsonized bacteria and dusts in the lung (Arredouani et al. 2005). Some receptors recognize carbohydrate moieties on microorganisms. They are the mannose receptor (MR) (-C-type lectin superfamily), galectin 3 (Stahl 1992), and dectin 1 which are important receptor for the phagocytosis of fungi (Herre et al. 2004). In mammals, both dectin-1 and complement receptor 3 (CR3) recognize β-glucans. The β-glucan recognition site on CR3 is distinct from the complement recognition site. It is

worth to note that in horseshoe crab, β-glucans mediate coagulation cascade, which is initiated on the fungi surface (ref. in Dzik 2010).

The Toll/TLR pathway is already present in the anthozoan cnidarians (Miller et al. 2007). Although TLR-mediated immunity developed independently in arthropod and mammals, similarities between proteins taking part in mammalian and *Drosophila* signal pathways are obvious (Akira et al. 2006). TLRs are expressed by a range of immune cells (dendritic cells, macrophages, and B cells) and also by nonimmune cells including epithelial cells (Kawai and Akira 2010). All TLRs, except for TLR3, recruit myeloid differentiation primary response protein 88 (MyD88) as an adaptor protein, crucial for the activation of NF-κB, which controls the expression of pro-inflammatory mediators (Kawai and Akira 2010) such as tumor necrosis factor-α (TNF-α), IL-1b, and IL-6. In TLR4 and TLR3 signaling, a second downstream pathway is involved. This pathway is mediated by Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) protein and results in delayed NF-κB activation and activation of type I interferons (Kawai and Akira 2010).

Another type of receptors used in phagocytosis are NLRs. The representatives of nucleotide-binding oligomerization domain-like receptors (NLRs) NOD1 and NOD2 are intracellular NLR that can induce a signaling cascade in response to pathogen recognition, resulting in the activation of NF-kB (Girardin et al. 2001; Martinon and Tschopp 2005). These receptors are involved in bacterial sensing as they recognize bacterial cell wall components, peptidoglycans. The cell wall of Gram-negative bacteria includes peptidoglycan structures containing meso-diaminopimelic acid, which can be recognized through NOD1 (Chamaillard et al. 2003). In contrast, NOD2 recognizes muramyl dipeptide, a component of peptidoglycans present in both Gram-negative and Gram-positive bacteria (Girardin et al. 2003; Inohara et al. 2003). It is worth to note that mutations in NOD2 receptors are associated with autoinflammatory diseases, such as Crohn disease and Blau syndrome in humans (Hugot et al. 2001).

Other members of the NLR protein family, NLRC3 and NLRP6, are negative regulators of TLR-induced NF-kB and MAPK signaling. Similarly NLRC5 is a negative regulator of both NF-kB and type I IFN signaling (ref in Cui et al. 2014).

Chemotactic receptors, PRRs, NLRs, the *r*eceptor for *a*dvanced *g*lycation *e*nd products (RAGE), and TLRs, sense damage-associated molecular patterns (DAMPs) named alarmins. Alarmins are released in response to cell death, following immune cell activation or matrix degradation (Bianchi 2007), and are strong inducers of sterile inflammation. Binding of alarmins to the PRRs leads to the activation of signaling cascades resulting in the activation of the transcription factor NF-κB, followed by the transcription of several pro-inflammatory genes (Bianchi 2007). Alarmins also promote the recruitment and activation of antigen-presenting cells. For instance, low molecular weight form of hyaluronan (HA) is synthesized under inflammatory conditions or is a product of the breakdown of high molecular weight HA (a component of the extracellular matrix.) This type of low molecular weight molecule brings about chemokine production via activation of macrophage TLR2 and TLR4 (Jiang et al. 2005). TLR2 and/or TLR4 are also activated by other

matrix components such as versican, heparan sulfate, fibronectin, tenascin-C (Piccinini and Midwood 2010), as well as soluble form of biglycan. This small leucine-rich proteoglycan increases TNF-α and macrophage inflammatory protein-2 (MIP2) levels during tissue injury or by excretion from activated macrophages (Schaefer et al. 2005). Recent evidence suggests that DAMPs may also play a role in the development of cancer; they act as a double-edged sword (Hernandez et al. 2016). DAMP group include also high mobility group box 1 (HMGB1, earlier described as HMG1 or amphoterin), a nonhistone chromatin-associated factor that regulates gene transcription (Lotze and Tracey 2005). It can induce the expression of TNF-α and tumor metastasis (Dumitriu et al. 2005; Ulloa and Messmer 2006) in jawed vertebrates. Analogues of HMGB1 have been reported both in jawless vertebrates and invertebrates (Müller et al. 2004). It was shown that stimulation of invertebrate hemocytes with LPS elevates expression of HMGB1 mRNA (Lin et al. 2013). The levels of HMGB1 have been found to be elevated in a number of autoimmune conditions (Venereau et al. 2016).

Macrophage Fc receptors and complement receptors internalize opsonized particles. FcγRIIA are equipped with a signaling domain, while FcγRI and FcγRIII need an adaptor protein to transmit the signal. Binding of opsonized particle to the receptor activates src kinases, which phosphorylate tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) of the cytoplasmic tail FcγRIIA or the adaptor dimer of FcγRI and FcγRIII receptors. The signal from activated Fc receptors leads to the activation of Rho GTPases that results in cytoskeletal changes and phagocytosis. Three complement receptors CR1, CR3, and CR4 are expressed on macrophages. CR1 binds opsonized particle, and CR3 and CR4 bind C3bi and, thus, help internalization of particles (Aderem and Underhill 1999). Dissimilar to FcγR, complement receptors need additional stimulation to start phagocytosis. It is worth to note that phagocytosis by this path is not dependent on the production of ROS (Wright and Silverstein 1983).

5.4 Phenotypes of Vertebrate Macrophages

Phagocytosis, bacterial killing, and homeostatic functions (wound repair, matrix remodeling) demand different macrophage functions. Two macrophage phenotypes in teleosts correspond to the rodent M1 and M2 activation states. Fish macrophages of the M1 phenotype are able to rapidly kill pathogens by engulfment and production of toxic reactive intermediates (Neumann et al. 2000). These macrophages produce cytokines, chemokines, and lipid mediators that potentiate and fine-tune the inflammatory and adaptive immune responses. M2a type of activation is confirmed by an enhanced arginase activity (Joerink et al. 2006) and the occurrence of fish IL-4 and IL-13 homologues (IL-4/IL-13A and IL-4/IL-13B) with low homology to mammalian counterparts (Li et al. 2007; Ohtani et al. 2008). Similar anti-inflammatory roles of glucocorticoids, immune complexes, IL-10, and TGF-β have been demonstrated in teleosts (Castro et al. 2011; Grayfer et al. 2011a, b). The

extremely high degree of conservation of TGF-β1 suggests that signaling function of this cytokine may have been preserved over time (Dzik 2014). Different macrophage phenotypes in the teleost show conservation of inflammatory and healing functions through vertebrate phylogenesis.

The main cytokine inducing M1 phenotype in mammals is IFN-γ (type II of interferon), which is secreted transiently by NK cells. Genes of type I and type II interferons and their receptor genes were cloned from teleost fishes (ref. in Robertsen 2006). M1 type of rodent macrophages produces free radicals, nitric oxide, and superoxide anion. Similarly, superoxide anion production by NADPH oxidase has been observed in teleost macrophages following stimulation with pathogen-associated molecular patterns (PAMPs); recombinant cytokines such as TNF-α, IFN-γ, and CSF-1; and fish pathogens (ref. in Hodgkinson et al. 2015). This is in contrast to the mammalian CSF-1, which is considered to be an M2 stimulus, opposite to GM-CSF (Mills and Ley 2014). Classically polarized fish macrophages (the carp kidney macrophage model), like rodent macrophages, are characterized by high iNOS gene expression. NO production increases following exposure to a microbial stimulus, PAMPs, or pro-inflammatory cytokine stimulus (ref in Hodgkinson et al. 2015).

In mouse macrophages arginase activity is stimulated after treatment of cells with IL-4, cAMP, or TGF-β (Mills et al. 2000), and this enzyme is a marker of M2 type of activation. Two arginase isoforms, arginase-1 (Arg-1) and arginase-2 (Arg-2), have been found in mammals, with guinea pig alveolar macrophages expressing arginase-1 (Dzik et al. 2004) and rat alveolar macrophages expressing both of them, but in terms of total activity, arginase-1 appears to be the major enzyme (Klasen et al. 2001). Bony fishes express *ARG-1* and *ARG-2* also, but both isoforms possess mitochondrial targeting sequences (Joerink et al. 2006) suggesting the mitochondrial localization for these two isoforms. Similar to rodent M2 macrophages, polarization of common carp macrophages into M2 phenotype with cAMP results in a high upregulation of ARG-2, but not ARG-1 gene expression (Wiegertjes et al. 2016). Also, like in mammals, fish macrophages are detected in healing wounds (ref in Yamaguchi et al. 2015). Divergence pro-inflammatory and homeostatic functions have been observed also in phagocytes of lamprey (jawless vertebrate) (Havixbeck et al. 2014).

In mammals, inhibition of expression of pro-inflammatory cytokines by IL-10 through its IL-10R1 and IL-10R2 receptors leads to activation of STAT3, thus dumping M1 and Th1 responses (Ferrante and Leibovich 2012). The anti-inflammatory function of fish IL-10 was confirmed by observed downregulation of both IFN-γ-stimulated ROS production and inflammatory gene expression in monocytes of goldfish (Grayfer et al. 2011a, b). The analysis of cladograms reveals that fish receptors for IL-10 are grouped independently of the amphibian, avian, and mammalian respective receptors (Monte et al. 2015).

5.4.1 Classification of Macrophages

Macrophages are the first line of defense against pathogens due to their microbicidal activity and secretion of pro-inflammatory cytokines in order to strengthen the cell-mediated adaptive immunity. Macrophages are involved in response to parasite, allergy, tissue remodeling, and wound healing. Some of these activities (inflammatory and wound healing) are antagonistic, and distinct molecules from the milieu exert the proper immune response. Pro-inflammatory ("classical") activation of macrophages (Mills et al. 2000) depends on the secreted molecules of activated T helper 1 (Th1) CD4+ lymphocytes or natural killer (NK) cells and in particular of interferon-γ (IFN-γ) and of other pro-inflammatory agents such as tumor necrosis factor-α (TNF-α) and bacterial lipopolysaccharide (LPS). In contrast interleukin-4 and IL-13 are the major inducers of the "alternative activation." "Classically" and "alternatively" activated macrophages have been designated as "M1" and "M2" macrophages, respectively, by analogy to the Th1/Th2 division of labor of CD4 helper T cells (Mills et al. 2000). M2-polarized macrophages are further subdivided into M2a (elicited by IL-4 or IL-13), M2b (following stimulation by immune complexes in the presence of a Toll-like receptor ligand), and M2c (when exposed to anti-inflammatory stimuli such as glucocorticoid hormones, IL-10, or TGF-β) (Martinez et al. 2008). In addition to M1 and M2 macrophages, regulatory macrophages (RMs) have recently emerged as an important population of cells that play a pivotal role in limiting inflammation during innate and adaptive immune responses (Mosser and Edwards 2008). Similar to M2 macrophages, RMs produce high levels of IL-10; however, unlike M2 cells, they do not contribute to the production of extracellular matrix and express high levels of the costimulatory molecules CD80/B7-1 and CD86/B7-2 (Edwards et al. 2006). Studies in mice have demonstrated a crucial role for both IL-4/IL-13 and IFN-γ signaling pathways in the induction of RM (Gallina et al. 2006; Sinha et al. 2005). Furthermore, they have shown that regulatory macrophages concurrently express nitric oxide synthase and arginase suggesting that they have a distinct activation phenotype (Gordon and Taylor 2005).

This classification of macrophages does not fully represent the complexity of the transitional states of macrophage activation in response to different microenvironments. A more flexible classification has been suggested recently by mouse studies in which macrophages are considered as part of a continuum having a range of overlapping functions and in which classically activated, wound-healing, and regulatory macrophages occupy different points of the spectrum (Mosser and Edwards 2008). However, also this classification does not take into account the role of macrophages during development encompassing embryonic (Rae et al. 2007) and wound-healing macrophages (Mosser and Edwards 2008) as well as irreversibly differentiated osteoclasts (Edwards and Mundy 2011). The differentiation of macrophages is profoundly influenced by the microenvironment, although there is considerable plasticity between distinct cell types.

5.4.2 *Plasticity and Polarization of Macrophages*

Macrophages have a plastic profile of gene expression that depends on the type of the stimulating agents, their concentration, and time of exposure to these agents (Biswas et al. 2008; Stout and Suttles 2004). Exposure of M2 macrophages to TLR ligands or IFN-γ (Modolell et al. 1995) results in expression of M1-associated genes. Gene expression plasticity is common when polarization is carried out in experimental conditions (Stout et al. 2005; Mylonas et al. 2009; Wells et al. 2003). This phenomenon proves that macrophages can adopt their response to different microenvironmental stimuli such as chemotactic signals or chemokines that guide macrophage migration, stimulate phagocytosis, and allow them to interact with different T-cell subsets. Stimulation of macrophages with opposite cytokines, such as IFN-γ and IL-4, induce signal cascades resulting in enhancement or inhibition of different genes at the transcriptional or posttranscriptional level. Macrophages may revert to their original status after the cytokine signaling terminates. It was found that most Th1 and Th2 cytokines do not stably induce distinct subsets of macrophages, but rather promote transient responses that return to basal levels in a day.

Observed heterogeneity of macrophages in cancer, viral infection, or other pathologies proves that the plasticity of macrophages is a physiological process serving for a defense against pathogens and transformed cells. Tumor-associated macrophages (TAMs) are located both in primary and secondary tumors; they were regarded as an archetype of the M2 type (Wynn et al. 2013). However, TAMs present markers of both the M1 and M2 types of macrophage polarization (Doig et al. 2013). TAMs reveal several properties that enhance cancer progression and metastasis. Although all of these macrophage subpopulations express the macrophage canonical markers CD11b, F4/80, and CSF-1R, as well as absence of Gr1 (Ly6G), a particular phenotype depends on signals from microenvironment. As a result, these subpopulations express various markers depending on whether they are invasive, activated, immunosuppressive, angiogenic, or metastasis-associated macrophages (Cassetta et al. 2011).

Monocyte-derived macrophages (MDMs) polarized in vitro toward M1 or M2a strongly inhibit HIV-1 replication and production, although different steps of the virus life cycle are influenced (Cassol et al. 2009). M2a-polarized macrophages "HIV-1-transmitting macrophages" showed to be highly phagocytic. They were able to transfer efficiently HIV-1 to $CD4^+$ T cells (Cassetta et al. 2011). In contrast, M1-MDM demonstrated the phenotype of "HIV-1-resistant macrophages" as they showed very low levels of virus replication. These "HIV-1-resistant macrophages" were low phagocytic and characteristic for the intracellular expression of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A (APOBEC3A). APOBEC3A has been found to be associated with an antiviral effect in monocytes (Peng et al. 2007) as well as in MDMs (Berger et al. 2011; Koning et al. 2011)

5.4.3 *Polarization of Human Macrophages*

Martinez et al. (2006) obtained a gene expression profile of polarized human macrophages after differentiation of monocytes into mature macrophages in the presence of M-CSF. Monocytes differentiating in the presence of M-CSF presented upregulation of cell cycle genes, such as the cyclins A2, B1, B2, D1, D3, and E2 and CDCA 1, 2, 5, 6, and 7. These observations point to the proliferation potential of monocytes according to the earlier reports on human monocyte/macrophage proliferation (Cheung and Hamilton 1992; Bischof et al. 2000). M1 polarization (LPS plus IFN-γ) of human macrophages was associated with fundamental changes in the transcriptome, while M2 polarization (IL-4) resulted in minimal alterations in gene expression. M1 and M2 macrophages differ in respect of chemokine expression. In addition to well-known CXCL10 for M1 and CCL17 for M2 cells, CCL13, CCL14, CCL17, CCL23, and CCL26 were expressed in M2 cells while CCL8, CXCL13, CCL15, CCL19, and CCL20 in M1 cells. The chemokine CCL20 is connected to autoimmune diseases in humans on account of its capability to attract Th17 cells to a site of inflammation (Comerford et al. 2010). High expression of nucleotide receptors in M2 macrophages endows M2 cells with sensors for tissue damage (Martinez et al. 2006). The UDP-glucose receptor GPR105 that regulates leukocyte chemotaxis in response to UDP-Glc (Lee et al. 2003) appeared to be among the most highly regulated genes in response to IL-4. Expression of the GPCR was found in M2 mouse macrophages as well. Alternatively activated human macrophages are also characterized by increased expression of fibronectin (Gratchev et al. 2001), which is involved in cell adhesion and migration processes during embryogenesis, wound healing, blood coagulation, and metastasis.

When gene and protein repertoires were compared between resting macrophages from the human and mouse, a set of molecules highly expressed representing a consistent 87-gene macrophage molecular signature (biased by arbitrary cutoff) were identified (Martinez et al. 2013). One group covers the genes involved in redox control and antioxidative defense. Peroxiredoxin 1 (PRDX1), aside a well-known antioxidant role, can induce TLR4 signaling and activation of NF-kB that cause the secretion of TNF-α and IL-6 (Riddell et al. 2010). The selenoproteins, glutathione peroxidase (GPX1), and thioredoxin reductase (TXNRD1) (Reeves and Hoffmann 2009) have recently been linked to the production of endogenous activators that mediate the peroxisome proliferator-activated receptor γ (PPARγ)-dependent switch from the M1 to the M2 phenotype in the presence of IL-4 (Nelson et al. 2011). PPARγ is a fatty acid sensor that plays a critical role in atherosclerosis and glucose metabolism (Chawla et al. 2001). Among lectins the galectin-3 is amply expressed in macrophages (Liu et al. 1995). It plays a role in regulation of inflammation and fibrosis (Henderson and Sethi 2009) and is critically involved in alternative macrophage activation because of a positive feedback loop leading to sustained PI3K activation (MacKinnon et al. 2008). Genes coding for proteins involved in cell adhesion, integrin β2 (ITGB2, CD18) (Jones et al. 1998), and chemotaxis, S100A10 and S100A4 (Phipps et al. 2011; Li et al. 2010), were

significantly expressed in resting macrophages both in human and mouse. As described above pattern of gene expression shows that highly expressed genes in resting macrophages of both species belong to inflammatory as well as homeostatic functions that correspond to M1 and M2 type of macrophage activation, respectively.

Comparison of expression of particular polarization markers between human and mouse indicates that about 50% of them contribute to one species and not to the other (Martinez et al. 2013). For example, well-known markers of alternative activation of mouse macrophages, arginase-1, Fizz1, MMP1, and Ym1, were not IL-4 induced (Martinez et al. 2006) or IL-13 induced (Scotton, et al. 2005) in the human macrophages. On the other hand, some molecules, fibrinoligase (F13A1), and platelet-derived growth factor C appeared to be only the markers of human macrophage alternative activation. Finally, some receptors with long evolutionary history are upregulated both in M2 human macrophages and mouse M2 macrophages. There are the mannose receptor 1 (Stein et al. 1992), the macrophage scavenger receptor 1 (Cornicelli et al. 2000), the C-type lectin-like receptors (CLR) dectin-1 (Willment et al. 2005), and the DC-SIGN (CD209) (Relloso et al. 2002). Similarly, transglutaminase 2 (TGM2), the multifunctional enzyme, was found to be the activation marker for mouse and human M2 macrophages and monocytes. TGM2 is suggested to take part in metastasis, fibrosis, and wound healing (Mehta et al. 2010; Grenard et al. 2001). TGM2 can also activate TGF-β, inducing anti-inflammatory and profibrotic cytokines (Mehta et al. 2010). All these roles of TGM2 parallel function of M2 macrophages. Analysis of TGM2 expression in macrophages and monocytes from asthma patients showed that TGM2 is expressed by human alternatively activated macrophages in vivo (Martinez et al. 2013). LPS is the most potent stimulant of M1 macrophages derived from cell wall components of Gram-negative bacteria. In human, but not mouse macrophages, LPS induces STAT4 and its target gene IRF-4 (Lehtonen et al. 2005). Possibly, such regulatory divergence is one of factors contributing to phenotypic discrepancies among species in macrophage response.

5.4.4 Molecular Mechanisms Underlying the Polarization of Macrophages

Wang et al. (2014) summarized molecular events leading to macrophage polarization as follows: macrophage polarization and activity is tightly regulated by the balance between activation of STAT1 and STAT3/STAT6. M1 macrophage polarization is promoted by a predominance of NF-kB and STAT1 activation. In contrast, IL-4/13 and IL-10 increase M2 macrophage polarization due to a predominance of STAT3 and STAT 6 activation. Kruppel-like factor 4 (KLF-4), a downstream of STAT6, takes part in the promotion of M2 macrophage functions suppressing the NF-kB/HIF-1a-dependent transcription. Hypoxia-inducible factors

(HIFs) are upregulated in hypoxia conditions in inflammatory sites to which macrophages are recruited (ref. in Wang et al. 2014). IL-4 induces not only c-Myc, which controls the expression of a subset of M2-associated genes, but also the M2-polarizing IRF-4 pathway to inhibit IRF5-mediated M1 polarization. IL-10 promotes M2 polarization through the induction of p50 NF-κB homodimer, c-Maf, and STAT3 activities. The peroxisome proliferator-activated receptors PPARδ and PPARγ control different aspects of M2 type activation and oxidative metabolism (ref. in Wang et al. 2014).

MicroRNAs modulate macrophage polarization through suppressor of cytokine signaling 1 (SOCS1), transcription factors CEBPα and CEBPδ, and Pknox1 (ref. in Wang et al. 2014). Among microRNA, miRNA-155 is a key molecule directing macrophage polarization toward M1 phenotype (ref. in Wang et al. 2014). In insects, microRNA is known to regulate the expression of immunity-related genes for pattern recognition, cellular responses, antimicrobial peptide synthesis, and intracellular signal transduction (Zhang et al. 2014). Similarly, immune challenge induces the expression of immunity-related miRNAs, which might modulate the immune response such as redox reaction, phagocytosis, and apoptosis in mollusk hemocytes (Zhou et al. 2014).

Recently, a great body of evidence have documented that polarization of macrophages is regulated by specific epigenetic mechanisms of histone modifications. Investigation of this mechanisms helps to explain how polarized macrophages acquire and maintain their activation phenotype. According to Kittan et al. (2013), the histone (H3K4)-methyltransferase MLL and demethylase KDM6B are upregulated in M1 macrophages while resting, and M2 macrophages are characterized by DNA methyltransferases and histone deacetylases. Further, in mice, marker genes of M2-macrophages are regulated by reciprocal changes in histone H3 lysine-4 (H3K4) and histone H3 lysine-27 (H3K27) methylation; and the latter methylation marks are removed by the H3K27 demethylase *Jumonji domain containing 3 (Jmjd3)*. Continuous IL-4 treatment brings about the decreased H3K27 methylation at the promoter of M2 marker genes and a concomitant increase in Jmjd3 expression. IL-4-dependent Jmjd3 expression is mediated by STAT6, a major transcription factor of IL-4-mediated signaling. After IL-4 stimulation, STAT6 level increases and STAT6 binds to consensus sites at the Jmjd3 promoter. Increased Jmjd3 contributes to the decrease of H3K27 dimethylation and trimethylation (H3K27me2/3) markers as well as the transcriptional activation of specific M2 marker genes. The decrease in H3K27me2/3 and increase in Jmjd3 recruitment were confirmed by in vivo studies using a *Schistosoma mansoni* egg-challenged mouse model, a well-studied system known to support an M2 phenotype (Ishii et al. 2009). Jmjd3 is a gene essential for M2 macrophage polarization in response to helminthic infections (Satoh et al. 2010). These authors also identified Irf4 as a Jmjd3 target gene, a key transcription factor that controls M2 polarization. More recently, Zhang et al. (2011) observed that genes with a potential for increased/decreased expression after macrophage polarization (i.e., IFN-γ, IFN-α, and IL-4) were generally enriched for cytokine-induced H4 acetylation (H4ac) (Zhang et al. 2011). Mitogen-activated protein (MAP) kinases were

shown to be a central signaling pathway for the macrophage polarization, and recent studies highlight the role of MAP kinases in histone modification (Zhang et al. 2011).

5.5 Natural Killer Cells

5.5.1 Definition and Classification

Macrophage partners from a phylogenetic standpoint of the development of innate immune system are lymphoid cells named natural killer (NK) cells (Janeway and Medzhitov 2002). After recognition more than 40 years ago, NK cells were characterized as innate immune lymphocytes creating the first line of defense against a variety of infections (viral, bacterial, protozoan, helminth, and fungal) and performing cancer surveillance and controlling cancer development through the recognition and direct killing of target cells and/or production of a variety of bioactive molecules without prior sensitization (Lodoen and Lanier 2006; Langers et al. 2012; Horowitz et al. 2013).

Further investigations of the NK cells revealed their population heterogeneity and rather sophisticated and intricate mechanisms of action. In the light of recent discoveries supported by a functional and genomic analysis data, a new characteristic of NK cells has been proposed (Walzer et al. 2007; Vivier et al. 2011; Hazenberg and Spits 2014). The uniform nomenclature for innate lymphoid cells has been presented by Spits et al. (2013). Currently the NK cells are considered to be members of the innate lymphoid cell (ILC) family originated from a common lymphoid progenitor (CLP). The CLP differentiates into a committed ILC precursor under the influence of transcription factor NFIL3 regulating expression of transcriptional repressor inhibitor of DNA-binding 2 (Id2) protein and DNA-binding protein Tox (Eberl et al. 2015). Natural killer cells, called also conventional NK cells (cNK), develop from ILC through an NK cell precursor in a manner dependent on Eomesodermin (Eomes) and T-bet transcription factors, whereas all other ILCs arise from a common helper innate lymphoid precursor (CHILP) developing from ILC under influence of DNA-binding proteins Id2 and PLZF and transcription factor GATA-3 to a committed innate lymphoid cell precursor (ILcP). ILcP is giving rise to class 2 and 3 of ILCs. On the basis of their phenotypic and functional criteria, NK cells are included into class 1 ILC. Recently, besides NK cells, the class 1 ILCs has been broaden by the inclusion of subset of ILC1 cells that share common ILC precursor with NK cells, but for their development do not require Eomes (Spits et al. 2013; Seillet et al. 2016). The particular role of two conserved families of T-box transcription homologous factors T-bet and Eomes in NK cell subset development, maturation, and function has been characterized by Simonetta et al. (2016). Models for ILC lineage commitment are described by Serafini et al. (2015) and Artis and Spits (2015).

5.5.2 Human NK Cell Population

Traditionally, human NK cells have been defined phenotypically by their surface expression of CD56 and CD16 molecules and lack of CD3 molecule. Two phenotypes of human NK cells were recognized: circulating in peripheral blood (PB) $CD16^{+}$ $CD56^{dim}$ and $CD16^{-}$ $CD56^{bright}$ preferentially recruited into tissues through expression of CCR7, the homing receptor for secondary lymphoid compartments. Presently, on the basis of differential phenotypic and functional properties, the lymphocytes belonging to ILC class 1 have been further separated into subsets including circulating and various tissue-resident NK cells. The latter group is still broadening and at present includes lymph node, salivary gland, liver, spleen, skin, mucosa, and uterine NK cells, which reveal great cellular and functional plasticity (Shi et al. 2011; Tang et al. 2016; Björkström et al. 2016). The majority of peripheral blood and spleen NK cells belong to $CD56^{dim}$ and only 5–15% to $CD56^{bright}$ subset, whereas secondary lymphoid tissue NK cell population comprises mostly of $CD56^{bright}$ subset (Ferlazzo et al. 2004).

Certain NK subsets have been classified as "regulatory" due to their pleiotropic functions (Spits and Di Santo 2011). This includes mucosal NK cells, not cytotoxic but producing cytokines including interleukin 22 (IL-22) and maintaining epithelial-cell barrier function (Cella et al. 2009), and uterine NK cells, representing 50–70% of the human decidual lymphocytes that promote tolerance and appropriate placenta and fetus development (Hanna et al. 2006; Manaster and Mandelboim 2010), as well as liver NK cells playing essential role in liver immune tolerance (Crispe 2014). Immunosuppressive TGF-β and IL-10-producing NK cells have also been described in mice and humans (Maroof et al. 2008). Regulatory function of NK cells in most cases is due to IL-10 secretion but can also rely on the killing of regulatory T cells (Zhang et al. 2006).

The functional diversity of NK cells have been also described using the expression of CD27, the TNF receptor (TNFR) family considered as marker of mature cell populations with low cytotoxic potential. Due to that classification, the regulatory NK cells belong to $CD56^{bright}$ $CD27^{+}$ NK cells, whereas NK-tolerant subset is $CD56^{bright}$ $CD27^{-}$. NK cytotoxic subset belonging mainly to $CD56^{dim}$ NK cells is also $CD27^{-}$ (Vossen et al. 2008; Fu et al. 2014).

Similarly to macrophage phenotype polarization under influence of various cytokines and environmental factors (Kittan et al. 2013), the related pattern was also proposed for NK cells. Beside the regulatory NK cells induced by IL-2 and releasing IL-10 and TGF-β and NK cells secreting IL-22, two other subsets have been differentiated: NK1 induced by IL-12/IL-18 and secreting IFN-γ, TNF-α, and IL-10 and NK2 developed under the influence of IL-4 and producing IL-5 and IL-13. Both subtypes are present in peripheral blood and lymph nodes (Peritt et al. 1998; Deniz et al. 2002). The specific phenotype of NK17/NK1 cells was isolated from normal human PB after activation with IL-2 and from cerebrospinal fluid patients with multiple sclerosis. The cells express $CD56^{+}$ and $CCR4^{+}$ and produce IL-17 and IFN-γ (Pandya et al. 2011).

It is obvious that recognition and understanding of the NK and other ILC1 cells heterogeneity concerning their phenotype, development pathways, and effector functions are necessary to develop the novel therapeutic strategies.

5.5.3 Origin and Development of NK Cells

The better understanding of the distribution and range of functions of NK cells led to the question what is the origin of NK cell subsets circulating in blood and tissue specific. The major role in NK cell development belongs to bone marrow creating the proper environment for NK cell propagation and their commitment at periphery. Discovery of thymic NK cells with different molecular markers and functions that are localized intra-thymically and in secondary lymphoid organs (SLT) revealed the possibility that bone marrow-derived pro-NK cells after entering circulation and extravasation into SLT may develop into both $CD56^{bright}$ and $CD56^{dim}$ NK cells, with former remaining in the SLT and later returning into circulation (Di Santo and Vosshenrich 2006; Freud et al. 2006). The pro-NK cells have been also found in mucosa-associated lymphoid tissue of the gut (Caligiuri 2008; Klein Wolterink et al. 2010).

Recently the NK lineage-restricted progenitor (NKP) was identified, and the presence of the NKP in human bone marrow, fetal tissues, umbilical cord blood, and adult tissues (tonsils) has been confirmed. It has to be noticed that in hematopoiesis NKP is located downstream of CLP and generates in vitro and in vivo only mature functional NK cells (Renoux et al. 2015). This puts a new light on the developmental pathways of NK and other innate lymphocytes.

As it was mentioned earlier, the development, maturation, and function of NK cells are under control of the two T-box transcription factors, T-bet and Eomes, that are constitutively expressed. Both factors undergo modulation and are required for surviving and differentiation of peripheral NK cells. It has been shown that cytotoxic $CD56^{dim}$ NK cells express lower levels of Eomes and higher of T-bet than producing cytokines $CD56^{bright}$ subtype (Knox et al. 2014). Investigations of various populations of circulating and tissue-resident NK cells revealed different T-box transcription factor expressions and distinct developmental pathways. Liver- and skin-resident cells depend on T-bet for development and do not express Eomes, whereas uterine and salivary gland NK cells express high levels of Eomes and their development is not T-bet dependent. Collected data reveal that both T-bet and Eomes regulate in cooperative manner the cytokine production in NK cells, whereas T-bet appears to be a key factor of their cytotoxicity (Daussy et al. 2014; Juelke and Romagnani 2016; ref in Simonetta et al. 2016). Comprehensive review concerning murine and human NK cell development and differentiation and all three ILC groups characteristics have been published elsewhere (Yu et al. 2013; Luetke-Eversloh et al. 2013; Diefenbach et al. 2014; Juelke and Romagnani 2016).

5.5.4 NK Licensing/Education Mechanisms

Terminally differentiated effector NK cells, during the last maturation step, express inhibitory receptors and are able to perform induced cytokine production and cytotoxicity. Differentiation and acquisition of the most potent effector functions of NK cells called "arming" is accompanied by the expression of specific markers (CD57) and killer immunoglobulin-like receptors (KIRs). After differentiation from hematopoietic progenitors, NK cells acquire their function in two processes that reduce the responsiveness to self-cells referred as "licensing" and "education." One mechanism requires recognition of self-ligands by inhibitory receptors KIR and CD94/NKG2A that recognize allelic variants of the MHC class I ligands (Yokoyama and Kim 2006; Sullivan et al. 2008; Fauriat et al. 2010). Another process is the result of the reducing of the responsiveness to self-molecules in NK cells stimulated by activating receptors without the interaction with inhibitory receptors. The human NK cell education by activating KIR receptors was proven in cell lacking the inhibitory KIRs. However, the recent investigations by Pradier et al. (2016) revealed that there is no direct interaction between the T-bet/Eomes modulation and the KIR expression during NK cell maturation. NK cells lacking inhibitory receptors are hyporesponsive and recognized as "uneducated" or "disarmed" (Anfossi et al. 2006; Comerford et al. 2007).

It has to be pointed out that further adjustment of the level of responsiveness may depend on cytokines and/or other factors, e.g., inflammation or cancer development in the environment of NK cells (Juelke et al. 2009; Thomas 2015; Kadri et al. 2016). Moreover, the NK cells may initially respond, but later became hyporesponsive after exposure to MHC-deficient environment, as it was documented in mice model (Joncker et al. 2010). On the other hand, the uneducated NK cells without inhibitory receptors are capable of becoming activated under certain conditions. And since they lack inhibition in contact with self-cells expressing MHC class I, they may be more beneficial in the clearance of these cells (Bryceson et al. 2006a; Tarek et al. 2012; Thomas 2015).

Data providing support for possibility of continuum of differentiation in vivo human $CD56^{(bright)}$ NK cells to $CD94^{(low)}CD56^{(dim)}$ phenotype have been also presented (Romagnani et al. 2007; Yu et al. 2010). For these reasons the theory of dynamic "rheostat" was developed based on the concept that NK responsiveness varies quantitatively, instead of approaching only two states (hyporesponsive versus responsive). Using the mouse model, it was shown that stimulatory and inhibitory signals are integrated in each NK cell depending on the expression of receptors and encountering of neighboring cells, and the responsiveness state is tuning quantitatively dependent on the strength of the inhibitory input and the net stimulation (Joncker et al. 2009; Brodin and Höglund 2008; Brodin et al. 2009; Shifrin et al. 2014).

Recently developed "confining model" reveals that NK cell responsiveness depends on distribution of cell receptors and their association with adhesion molecules and cytoskeleton at the level of plasma membrane. The education

process affects the compartmentalization of NK receptors providing the efficient contacts and NK cell activation (ref in He and Tian 2016). The comprehensive reviews have been published summarizing the detailed information concerning the development and maturation of murine and human NK cells (Cortez et al. 2015; Vivier et al. 2016; He and Tian 2016).

Emerging knowledge about developmental origin and various pathways of differentiation influenced by education process, tissue localization, and other environmental factors, e.g., infections or cancer, is enriched by epigenetic reprogramming during clonal-like expansion of NK cells. Particular diversity is observed in peripheral tissues. Extensive review of human NK cell developmental diversity and differentiation circulating and peripheral tissue-resident NK cells, as well as developmental pathways of tissue-resident NK cells, is given by Björkström et al. (2016). According to accepted model of NK cell maturation in bone marrow or thymus/secondary lymphoid tissues followed by entering circulation and localization in peripheral organs, authors proposed that at an early stage of development the NK cell precursors may exit BM and through circulation enter respective tissues creating "tissue-specific" NK cells. Some studies showed that inhibitory receptor expression was mostly determined by genetic factors, while environmental influence affected activation receptors. Estimation of the phenotypes of human peripheral blood NK cell population and separate $CD56^{(bright)}$ and estimation of the $CD56^{(dim)}$ demonstrated high plasticity of the NK cells in healthy donors (Angelo et al. 2015).

5.5.5 *Innate Memory NK Cells*

"Innate memory" or "trained memory" concerns the ability of innate immune cells to develop the immune response depending on a previous infections or vaccination and has been observed in invertebrates and vertebrates (Quintin et al. 2014; Netea 2013). Induced immune response may be increased or tolerogenic and is mostly dependent on epigenetic and/or metabolic reprogramming (Blok et al. 2015; Töpfer et al. 2015). The review of the development of innate memory of monocyte/macrophage and NK cell is given by Töpfer et al. (2015) who summarized the main mechanisms involved in realization of that program in both cell types. This includes altered cytokine release and expression of receptors, e.g., PRR (Kleinnijenhuis et al. 2012) and epigenetic/metabolic reprogramming as it will be discussed later.

It has been revealed that monocyte/macrophages and NK cells can develop features that have been considered as typical for adaptive immunity, like antigen-specific long-lived memory responses. The "memory-like" NK cells undergo clonal expansion and reveal the effective anticancer and antiviral responses that are connected with epigenetic modifications (Della Chiesa et al. 2016). Increased responses to secondary infections, as well as secondary expansion and protective immunity after adoptive transfer of memory NK cells into naive animals, have been

observed (Sun et al. 2009). Trained immunity provides protection against reinfection in a manner independent on T-/B-cell adaptive response (Netea 2013). It has to be pointed out that trained immunity, although less specific than adaptive immunity, may create the cross protection to other pathogens (Netea et al. 2011). Min-Oo et al. (2013) summarized the three pathways of NK cell memory formation due to various experimental approaches performed on mice models. Obtained memory cells revealed quite distinct features considering active cytotoxicity or cytokine production, specificity, and secondary expansion in a manner depending on stimulating factors. That include (x) virus-induced memory (MCMV mouse cytomegalovirus), (xx) cytokine-induced memory, and (xxx) liver NK cell-restricted memory cells. Investigations of human NK cells confirmed possibility of similar pathways for memory cell development.

Virus-induced pathway in human has been described. NK cells infected with HCMV (human cytomegalowirus) reveal expansion of memory-like NK cells with CD94/NKG2C activating heterodimer receptor and expansion of $NKG2C^{+}$ subset. That was also observed in patients with HCV9 (hepatitis C), HBV (hepatitis B), HIV, EBV (Epstein–Barr), or hantavirus infections (Foley et al. 2012; ref in O'Sullivan and Sun 2015). The role of activating KIRs and NKG2C in regulating NK cell responses and promoting a memory-like response to certain viruses is discussed by Della Chiesa et al. (2015).

Many observations documented cytokine-dependent development mechanism of human NK cells. Pretreatment of both human $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets with IL-12, IL-15, and IL-18 cytokines resulted in their extensive proliferation and enhanced IFN-γ production after cytokine restimulation (Leong et al. 2014). Cytokine-induced memory-like NK cells (CIML) represented phenotypic differences including increased CD94, NKG2A, NKp46, and CD69 and reduced KIR and CD57 surface expression (Romee et al. 2012; Rölle et al. 2014). The role of monocytes as a source of IL-12 has to be pointed out (Leong et al. 2014). Current status of knowledge concerning the cytokine-induced memory-like (CIML) NK cells has been reviewed by Berrien-Elliott et al. (2015). Particularly interesting is the induction of memory-like human NK cells in response to haptens, initially recognized in a mice model of contact hypersensitivity reaction that will be discussed later.

5.5.6 NK Cell Receptors

Molecular mechanisms controlling and determining NK cell reactivity comprise the wide array of activating and inhibitory receptors that sense and react to environmental alterations caused by exogenous and endogenous cellular stress and cancer transformation. Understanding the integrated function of that signals requires recognition of the structure, spatial localization, and specific features of that components (Long et al. 2013).

Distinct phenotypes and functions of NK cell subsets are determined by the expression of NKR transducing signals from self- and altered-self-cells and/or environment. In the contrary to adaptive B and T lymphocytes, which rearrange their receptor genes somatically, the NK cells can recognize variable patterns of ligands and regulate or amplify accordingly their effector functions through mechanisms involving dynamic interaction among the transmembrane germline-encoded receptors (Lanier 2008; Vivier et al. 2008; Kumar et al. 2016; Sivori et al. 2014b). NKRs belong to a most rapidly evolving eukaryotic gene families, structurally unrelated, comprising many types: activating, inhibitory, cytokine, chemokine (chemotactic), and adhesion molecules. It has to be pointed out that receptors belonging to all types can activate or inhibit NK cells depending on the environmental milieu. That requires the simultaneous and often synergistic signal transduction from various receptors for realization of the final NK cell response (Long et al. 2013).

Classification of mammalian NKRs can be performed by various approaches. On the basis of physiological function and signaling motifs, the NKRs can be separated into activating and inhibitory groups. Majority of inhibitory receptors have immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic tails, while activating receptors possess cytoplasmic ITAM and positively charged amino acid molecule that associates with negatively charged molecule of adaptor proteins within the transmembrane domain. Signaling pathway of activating receptors is initiated by the activation of cytoplasmic kinases and protein phosphorylation that induce cytokine and chemokine production and the release of cytolytic granules. The inhibitory receptors inhibit the activating NKRs action by the association with the intracellular Src homology-2 (SH2) domain-containing phosphatases (ref in Lanier 2008).

The second approach is based on the recognition of extracellular protein domains encoded by different gene complexes including leukocyte receptor complex (LCR) located on chromosome 19, natural killer complex (NKC) located on chromosome 12, and genes encoding natural cytotoxicity receptors (NCR) on chromosome 6 (Bartel et al. 2013). It has to be noticed that in mammals the NKR genes vary from minimally polymorphic single-copy genes to polygenic and polymorphic multigene families with high level of complexity and significant interspecies variations.

5.5.6.1 Killer Cell Immunoglobulin-Like Receptors

Superfamily of inhibitory killer Ig-like receptors (KIRs) create a very large group with similar signaling pathway including ITIM inhibition motif (Long 2008; Watzl and Long 2010). ITIM belongs to evolutionary old molecules, but this motif underwent rapid evolution causing the high level of polymorphism and low conservation of KIR genes between animal species and human (Parham et al. 2012). KIRs recognize polymorphic antigen HLA molecules: HLA-A, HLA-B, HLA-C, and soluble HLA-G as well as non-MHC ligands (Moretta et al. 1996). The anergy

of NK cells and lack of their cytolytic activity depends on the level of HLA type I molecules or other ligands for inhibitory KIR receptors on target cells according to "missing self" hypothesis (Karre 2008). Processes compromising the HLA I expression, such as cancer transformation or viral or bacterial infections, make cells sensitive to NK cell attacks upon receptor engagement (Moretta et al. 2001). Recently the collaboration between TLRs and KIRs was confirmed. In addition, an inhibitory KIR, such as KIR3DL2, may induce NK cell activation, promoting TLR-stimulated response (Sivori et al. 2014a).

5.5.6.2 Natural Cytotoxicity Receptors

Activating NCR receptors NKp46, NKp44, NKp30, and NKp80 belong to the Ig-like superfamily and recognize ligands that include pathogen-derived molecules as well as non-MHC self-molecules expressed on stressed cells (Vivier et al. 2011). Activating NCRs play a critical role in the control of tumors and in eradication of viral, bacterial, and parasitic infections and are also involved in regulatory functions due to production of IFN-γ, TNF-α and other cytokines and chemokines, as reviewed by Hudspeth et al. (2013a, b) and Kruse et al. (2014). Downregulation of NCRs can be due to inhibition of their expression or function. Specific environmental conditions created by developing tumors are known to suppress associated NK cells in cooperation with tumor-associated macrophages (TAM) and other cells (Balsamo et al. 2013). NCR receptors that contain ITIM motif are inhibitory.

5.5.6.3 C-Type Lectin-Like Receptors

In NK cells the main group of CLRs belonging to NKG2x subfamily is coded mostly by single gene and forms heterodimers with CD94 molecule creating the five different molecular species including inhibitory NKG2A and NKG2B and activating NKG2C, NKG2E, and NKG2H. The NKG2C and NKG2E receptors require association with the signaling adaptor chain DAP12, which contains a tyrosine-based signaling motif (Wada et al. 2004). Some of these receptors (NKG2) recognize MHC or MHC-like molecules, whereas the others (NKRP1, NKRP1A, NKp80, NKp65) interact with corresponding C-type lectin-like ligands (Cooper et al. 2009; Bartel et al. 2013). Both an inhibitory NK cell receptor (CD94/NKG2A) and an activating receptor (CD94/NKG2C) recognize the nonclassical MHC class I molecule HLA-E (Wada et al. 2004; Braud et al. 1998).

Activating NKG2D receptor is present on majority of NK cells. It is coded by a single gene with little polymorphism, is not associated with CD94, and requires the ITAM-independent transmembrane adaptor DAP10 to initiate full cytotoxic activation of NK cells (Upshaw et al. 2006). This major immunosurveillance receptor can bypass signals transmitted through inhibitory NK receptors that allows its function as a "master switch" in determining the activation status of NK cells. NKG2D is important for NK cell-mediated immunity to viruses and tumors and has

roles in autoimmune diseases since it recognizes different ligands induced by stress or infection as stress-inducible surface glycoprotein MICA (MHC class I chain-related antigen A), and related molecules MICB and UL16-binding proteins (ULBP-1-5) (Obeidy and Sharland 2009).

The CLR NKp80 present on NK cells are also expressed on monocytes and macrophages and recognize activation-induced C-type lectin (AICL) contributing to reciprocal activation of NK and myeloid cells. Human NK cells express NKp80 and the NKp80 ligand AICL, creating an autonomous control of human NK cell activity (Welte et al. 2006). It has to be noticed that due to diverse ability of recognition of carbohydrates and other molecules such as lipids and proteins characteristic for transformed cells and pathogens, CLRs are included into the group of pattern recognition receptors (PRRs) (Dambuza and Brown 2015).

5.5.6.4 Fc Receptor CD16

Activating human NK cell Fc receptor CD16 (FcγRIIIa) belongs to Ig-like receptors and is present on essentially all $CD56^{dim}$ PB NK cells, while small number or no receptor is expressed by $CD56^{bright}$ NK cells. This low affinity receptor for Fc fragment of IgG molecules enables the antibody-dependent cellular cytotoxicity (ADCC) reaction and is also involved in a direct recognition and lysis of some virus-infected cells and tumor cells, independent of antibody binding (Mandelboim et al. 1999). CD16 signaling follows the ITAM-dependent pathway. In contrast to many activating receptors, which require costimulatory signals, activation of CD16 alone is sufficient to activate both cytotoxicity and cytokine production in resting human NK cells (Bryceson et al. 2006b).

5.5.6.5 Co-activating Receptors

Induction of direct cytotoxicity and secretion mechanisms in primary resting NK cells require synergistic signals from co-activating receptors (Bryceson et al. 2006a; Long et al. 2013).

The co-activating human NK receptors 2B4 (CD244), NTB-A, and CRACC (monomeric Ig-like type I transmembrane glycoprotein) are members of the SLAM (signaling lymphocytic activation molecule)-related receptors (Claus et al. 2008). The intracellular domain of these receptors contains immunoreceptor tyrosine-based switch motifs (ITSM). Under certain circumstances 2B4, NTB-A, and CRACC can also inhibit NK cell functions (Veillette 2006). Other co-activating NK cell receptors include DNAM-1 (DNAX accessory molecule-1), NKp80, CD2, and leukocyte function-associated antigen 1 (LFA-1) molecules (Carlsten et al. 2007).

The overview of NK cell-activating and NK cell inhibitory receptor signaling, ligands, and function is given by Lanier (2008), Bryceson et al. (2011), and Long et al. (2013).

5.5.6.6 Pathogen-Associated Molecular Patterns Recognizing Receptors

NK cells express wide repertoire PRR comprising receptors recognizing PAMPs, DAMPs, and tumor-associated molecular patterns (TAMPs). This group includes TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and cytosolic DNA sensors including cyclic GMP–AMP synthase (cGAS) receptors. PRRs recognize a variety of bacterial, viral, fungal, and parasitic pathogens, as well as endogenous danger signals. Reviews of the NK cell PRRs and their ligands are given by Souza-Fonseca-Guimaraes et al. (2012) and Cui et al. (2014).

Human NK cells express TLR2, TLR5, TLR9 and TLR3, and TLR7/TLR8 binding bacterial and viral antigens, respectively (Adib-Conquy et al. 2014). Cytosolic PRRs include NLRs that recognize endogenous danger signals including molecules exposed and/or released during cellular stress or damage, as well as various microbial products and aggregate to form inflammasomes (Li et al. 2013).

The receptor of advanced glycation end products (RAGE) belongs to multiligand PRRs and binds also DAMP molecules typical of damaged cells, particularly HMGB1 and S100 proteins. It also can act as an adhesion molecule (reviewed by Sessa et al. 2014). Expression of RAGE by NK cells and its stimulation of IFN-γ production have been documented in mice model of human cancer in vivo and in vitro (Narumi et al. 2015).

PRR can interact among themselves as well as with other cytokine and chemokine stimulating signals. Synergy of TLRs and activating KIRs and NCRs was recognized (Sivori et al. 2014a; Cui et al. 2014). The presence of PRR on majority of innate and adaptive cells allows a coordinated response to the same pathogen-derived product (Della Chiesa et al. 2014).

5.5.6.7 Cytokine and Chemokine Receptors and Adhesion Molecules

Cytokine and chemokine receptors and adhesion molecules play a significant role in development, differentiation, education, target recognition, and effector function of NK cells including development of innate memory (Watzl and Long 2010; Juelke et al. 2009).

Human NK cells express high-affinity activating receptors for cytokine IL-2 (IL-2R) (constitutively on CD56 ^{bright} phenotype) and IL-21 (IL-21R), both cytokine produced by T cells. IL-21 activation of NK cells requires co-activation by other cytokines or activation of CD16 receptor. IL-21 alone or with IL-2 differentially affects function of various subsets of NK cells (Wendt et al. 2007). Activating receptors IL-1R, IL-4R, IL-12R, IL-15R, IL-18R, interferon (IFN)-αR/IFN-βR, and IL-10R for monocyte-/macrophage-derived cytokines are also recognized (Vivier et al. 2008; Schulz et al. 2010). The IL-15, pro-survival for NK cells, is indispensable for their development activation and expansion into secondary lymphoid tissue

(Fehniger and Caligiuri 2001). That cytokine membrane bound on dendritic cells and macrophages is trans-presented to the IL-15R on NK cells (Waldmann and Tagaya 1999; Mortier et al. 2009; Huntington 2014).

IL-18, a pro-inflammatory cytokine, is a costimulatory cytokine that acts synergistically with IL-12 and IL-15 and is secreted by activated phagocytes (French et al. 2006). Stimulation of effector function of $CD56^{bright}$ NK subset by cytokines generally requires two signals, one, almost always obligatory IL-12 and second, derived by IL-1, IL-2, IL-15 or IL-18, or coming from other activating receptors including NKG2D and CD16 (FcγRIIIa) (Cooper et al. 2001b; Parihar et al. 2002; Bryceson et al. 2006b). The multifunctional cytokine IL-10 increases cytotoxicity of NK cells against autologous macrophages and stimulates expression of activating receptors in a manner dependent on the presence of IL-2 (Schulz et al. 2010; Park et al. 2011).

The TNF superfamily receptors and ligands is critical for building innate and adaptive immune responses against foreign pathogens and cancer by regulating cell death and survival in cells under NK cell attack and by providing up- or downregulatory effects on the killer lymphocytes themselves (Locksley et al. 2001). Various members of the TNF receptor superfamily are expressed on NK cells (Vujanovic 2011; Rakhmilevich et al. 2012). TRAF-binding TNF receptors, including CD27, CD40, 4-1BB (CD137), and GITR (CD357) molecules, promote cell activation, differentiation, and survival. Upon activation through CD16 or by IL-2 and IL-15 treatment, many NK cells express TNF receptor (TNFR) superfamily members 4-1BB, and glucocorticoid-induced TNFR-related (GITR) receptors, both of each may function as activating or inhibitory, acting synergistically with other receptors (Baessler et al. 2010; Barao 2012).

TGF-β belongs to major immunosuppressive cytokines that prevents autoimmunity due to its antiproliferative and anti-inflammatory properties. In NK cells TGF-β exerts a broad range of effects from development and homeostasis to suppression of mature NK cell anticancer cytotoxicity, mainly by inhibition of IL-15-induced activation (Viel et al. 2016). It also reduces the expression of various activating NK cell receptors, e.g., inhibits NKG2D expression at the level of transcription (Crane et al. 2010). Inhibition of NK cell cytotoxicity in vitro is dependent on their direct contact with myeloid-derived suppressor cells (MDSC) membrane-bound transforming growth factor-β (TGF-β) (Li et al. 2009).

Chemokines control NK cell movement and specific localization in proper microenvironment that determine their maturation and final differentiation as it was shown in mice (Bernardini et al. 2014). In humans, various subsets of NK cells have unique repertoires of chemokine receptors including CCR7 and CXCR3 homing receptors to secondary lymphoid organs ($CD56^{bright}CD16-$) and CXCR1, CX3CR1, and ChemR23 receptors for trafficking to inflamed tissues ($CD56^{dim}CD16+$) as well as other CXCR, CX3CR, and S1P5 families (Robertson 2002). Beside the crucial role in NK cell recruitment, cytokines are important mediators of cytolysis (Taub et al. 1995). The chemokine role in biology of NK cells is summarized by Maghazachi (2010).

Also adhesion molecules determine the trafficking pathways of various subsets of NK cells (Carrega and Ferlazzo 2012). Among the various types of integrins, NK cells constitutively express leukocyte function-associated antigen 1 (LFA-1) (αLβ2, CD11a/CD18) and VLA-4 (α4β1, CD49d/CD29) and other molecules including CD2, CD44, CD49e, and ICAM-1. $CD56^{bright}$ NK cells display high level of L-selectin, whereas $CD56^{dim}$ expresses mainly LFA-1 molecules (Morris and Ley 2004). Their role in NK cell activation is also considered. It has been shown that IFN-γ and TNF-α synergistically enhance NK cell cytotoxicity through stimulation of ICAM-1 expression on target cells and promoting their direct interaction with NK cells (Wang et al. 2012a).

The important new group of NK cell receptors is represented by the DNAX accessory molecule (DNAM-1, CD226) that belongs to adhesion molecules involved in recognition of ligands expressed by cancer and infected cells. Those ligands include nectin and similar molecules induced and regulated under different pathological conditions that provoke the cellular stress (Bottino et al. 2003; Chan et al. 2014a, b). Comparison of cytokine, chemokine receptors, and adhesion molecules expressed by $CD56^{bright}$ and $CD56^{dim}$ NK cells is given by Poli et al. (2009).

5.5.6.8 Paired Receptor and Ligand Theory

Recently the paired receptors and their ligands theory has been proposed as reviewed by Kuroki et al. (2012) and Bartel et al. (2013). The coexistence of activating and inhibitory receptors on NK cells requires careful adjustment of their action for maintenance of the proper homeostasis in health and disease. Such pairs of receptors recognize in similar manner extracellular self- and non-self-ligands but transduce opposite signals. They often share specific general structural features. As it was earlier described, inhibitory receptors have long cytoplasmic domain containing ITIM motif, whereas activating receptors poses short cytoplasmic domain with positively charged residue interacting with adaptor protein containing ITAM motif. Extracellular domains comprise conserved amino acid sequences, which bind the ligands with rather low affinity, in order of μM concentrations, with fast association/dissociation exchange. The matter of importance is that usually inhibitory receptor binds the same ligand with higher efficiency than activating receptor that allows the inhibitory receptors to maintain immunological tolerance by the recognition of self-ligands (Kuroki et al. 2012). It is evident that paired receptors, enabling direct balance between the activating and inhibitory signals, allow the tight regulation of immune system and disruption of this system ends in various pathologies including chronic infections, autoimmunity, and allergy.

5.5.7 *Mechanisms Controlling NK Cell Development and Function*

Modulation of NK cell development, migration to sites of immune activation, and effector function is realized by multiple synergistic feedback interactions requiring the cell–cell contact with other components of immune system (including dendritic cells, monocytes/macrophages, mesenchymal and endothelial cells, and different T lymphocyte subsets) and the cytokines and chemokines present in microenvironment (Sivori et al. 2014b).

Detailed description of all transcriptional and posttranscriptional mechanisms controlling the NK cell homeostasis is out of scope of that chapter and is already summarized in many published reviews. The point of interest, however, is to signalize the new achievements concerning the transcription factors and new epigenetic mechanisms controlling the NK cell biology, particularly in the view of search for new therapies.

5.5.7.1 Cell–Cell Contacts

Many data confirm the spatial and temporal manner regulation of the NK cell activating and inhibitory receptor signal transduction and function (Köhler et al. 2010). Observations have been made that size of the receptors and ligands for both activating and inhibitory receptors of NK cells may affect their response and that optimal spatial match is required for creating immune synapse (IS) and signal integration (Brzostek et al. 2010). Activation of NK cell cytotoxicity by proper ligands is inversely dependent on their length. The difference in size also determines segregation and co-localization of receptor/ligand complexes within the immune synapse created between NK and target cell (Köhler et al. 2010). Detailed studies of the nanoscale organization of immune synapses created by KIR2DL1 (inhibitory) and KIR2DS1 (activating) receptors on human primary NK cell lines documented importance of the size of assembled nanoclusters, including DAP12 within activating receptors, and sequences of transmembrane ligand and receptor chain effecting the phosphorylation and activation of cytosolic kinase ZAP-70 or phosphatase SHP-1 for downstream signaling pathways (Oszmiana et al. 2016). The general principle is that NK cells' inhibitory or regulatory responses depend on selective recruitment of signaling molecules into synapse with simultaneous exclusion of proximal intracellular activating proteins (Eissmann and Davis 2010).

Culley et al. (2009) investigated the molecular mechanism of activating receptor NKG2D and the inhibitory receptor NKG2A and have shown that activating process is dependent on F-actin reorganization providing symmetrical synapse polarized toward the target cell. Inhibitory receptors create synapses named "kinapses", with much shorter life time, by disturbing the symmetry and reversing the stop signal (Culley et al. 2009; Mace and Orange 2011). Dynamic disassembly of F-actin required for NK cytolytic function depends on coronin A (Coro-1A), the

actin regulatory protein, specific for hematopoietic cells that facilitates the delivery of cytolytic granules into the immune synapse. The localization of Coro-1A in macrophages has been also documented (Mace and Orange 2014). The multiple steps, amounting to 48, of the formation of functional NK cell lytic synapse during the three main stages, recognition-initiation, effector, and termination, have been identified and described (Orange 2008; Mace et al. 2014).

Another possibility of connections between cells is realized by membrane (or tunneling) nanotubes (MNTs) which create dynamic structures enabling long-distance transfer of signal molecules, subcellular organelles, and vesicles as well as spreading pathogenic factors such as viruses or prions (Davis and Sowinski 2008; Zhang and Zhang 2013). MNT reveals considerable heterogeneity in composition, formation, and functions depending on involved cell types. Formation of MNTs by human NK cells and their important role for NK cells activation have been documented (Chauveau et al. 2010). The MNTs in human NK cells are not open-ended and contain elements of cell cytoskeleton such as F-actin and often α-tubulin and are form between primary NK cells and NK cells and cancer-transformed cells, including monocyte-derived THP-1 cell line. Activation of NK cells with cytokines stimulates the MNT formation due to increased expression of NKG2D. Target cells connected by MNT could undergo lysis at distant location or after migration along the nanotube to reach a close contact with NK cell. Investigations of the nanotube junctions revealed accumulation of DAP10 and Vav-1, signaling adaptors, and mediator molecules associated with activating receptor NKG2D and its ligand MHC class I chain-related protein A (MICA) at intracellular synapse between NK and target cells. The presence of submicroscale immune synapses in MNT junctions of NK cells allowed to drive conclusion about MNT's role in propagation of cytotoxic reaction (Chauveau et al. 2010).

Similar regulatory effects are due to extracellular microvesicles (EV)/exosomes, released by normal and tumor cells, which transfer functionally active protein and lipid ligands and nucleic acids between cells (Andaloussi et al. 2013; McCoy-Simandle et al. 2016). EV/exosomes, composed of lipids, proteins, and genetic materials including mRNA, miRNA, other small RNA species, and long noncoding RNA (lncRNA), released by immune and nonimmune cells, affect immune responses in normal and pathophysiological processes (van der Grein and Nolte-'t Hoen 2014; Zaborowski et al. 2015). Human peripheral blood NK cells constitutively, independently from their activation level, secrete EV containing soluble FasL and perforin (Lugini et al. 2012). Exosomes released from infected or cancer cells can provide the NK cell activation or inhibition, depending on a different molecular composition. Activating exosomes contain TNF-α and induce expression of INF-γ and cytolytic activity in NK cells, and inhibitory exosomes may contain TGF-β1 and NKG2D ligands and inhibit NK cells cytotoxicity (Reiners et al. 2013).

An important mechanisms by which the NK cells can modify their phenotypic/functional properties is the process called trogocytosis. This phenomenon is under the control of NK cell-activating and NK cell inhibitory receptors and allows the NK cell to transfer target cell membrane components into its own membrane creating a new receptor interaction through a direct contact (Tabiasco et al.

2002). Various immune and cancer cells may participate in such an intercellular communication (Davis 2007). Both KIR and NCR receptors are involved in that action (McCann et al. 2007; Marcenaro et al. 2009, 2013).

5.5.7.2 Transcriptional and Posttranscriptional Regulation

The wide array of the transcriptional and posttranscriptional mechanisms that underlie the maturation, differentiation, and functional specialization of NK cells has been recently recognized and characterized (Lim and McKenzie 2015; Leong et al. 2016). The checkpoints in NK cell development, expression of NKRs, and gaining final functional competence is controlled by DNA-binding factors and chromatin modifications. Description of proposed transcription factors and epigenetic regulations in an early stages of NK cell development, as well as mature NK cell effector functions depending on perforin, granzyme B, IFN-γ, and TNF-α expression, is given by Cichocki et al. (2013). The same authors also proposed the models of "memory" NK cell generation during cytokine priming as compared with viral infection. Recent data reveal the role of T-bet and Eomes in the transcriptional regulation of NK cell function and effects of their anomalies on aging, inflammation, and cancer (Thomas 2015).

Cacalano (2016) summarized the key role of the signal transducer and activator of transcription (STAT)-3 factor in regulation of major functions of NK cells including development, activation, killing the target cells, and interactions with other components of immune system, particularly in response to cancer. During normal NK development and progression, STAT3 is not critical, but its depletion causes increase of cytotoxicity and cytokine production both in vitro and in vivo. STAT-3 activation in NK cell decreases the expression of activating receptors DNAM-1, NKp30, NKp44, and NKG2D as well as production of perforin A and granzyme B. Many investigations of gene targeting mouse models revealed importance of the expression of T box transcription factor Txb21 and IL-15Rα on monocytes for final differentiation of NK cells as well as negative checkpoint realized by FoxO1transcription factor for NK cell development and differentiation in mice and humans (Soderquest et al. 2011; Deng et al. 2015; Wang et al. 2016). Recently, the novel mechanism regulating the NK cell proliferation and survival was described that revealed the role of 2 KLF-2 (Kruppel-like factor 2) transcription factor in limiting of the expansion of NK cells in peripheral tissues and affecting migration pattern of mature NK cells by directing them to IL-15-rich niches through regulation of homing receptors subset (Rabacal et al. 2016).

Epigenetic mechanisms alteration of the DNA methylation, mainly CpG hypomethylation, were identified during NK cell activation. Treatment of human NK cells with methyltransferase inhibitor 5-azacytidine strongly suppressed their cytolytic activity, which was associated with decrease of granzyme B and perforin secretion and overexpression of inhibitory KIRs, whose promoter regions are highly methylated (Gao et al. 2009b). Active NK cells are characterized by high DNA demethylation, whereas resting NK cells reveal low level of demethylation

(Wiencke et al. 2016). Mono-allelic pattern of KIR gene expression during NK cell maturation depends on the level of DNA demethylation. Expressed alleles exhibit hypomethylation, while hypermethylation leads to inhibition of expression. The expression of major activating receptor NKG2D is suppressed by methylation in the promoter region of gene and by deacetylation of histone 3 (ref in Schenk et al. 2016). Inhibitors of histone deacetylase impair NK cell cytotoxicity due to suppression of both, IL-2 activation and expression of activating receptors NKp30 and NKp36 (Ogbomo et al. 2007). Epigenetic alterations affecting the development and regulation of NK cells and the influence of external stimuli are reviewed by Schenk et al. (2016).

Important regulatory role for microRNAs (miRNAs or miRs) in maintaining NK cell homeostasis and controlling their effector function opens a new regulatory approach to NK cell biology. Summary of the effects of miRNAs on NK cell development, maturation, and effector function, particularly as control factors of oncogenic or tumor suppressor activity, is documented by Bezman et al. (2010) and Beaulieu et al. (2013).

The investigations of human NK cell miRNA transcriptome are summarized by Sullivan et al. (2013) who also presented data concerning the role of specific miRNAs involved in NK cell development, survival, maturation, and function including cytotoxicity and cytokine activation in health and disease. The further development in that field using the new approaches and techniques enables to recognize a novel miR profile of NK cells from different localizations. The miR-181 and miR-362-5p are considered as major promoter of NK cell functions and miR-583 as a negative regulator of NK cell differentiation due to silencing IL-2Rγ expression (Cichocki et al. 2011; Yun et al. 2014; Ni et al. 2015). MiR-1245 has been recognized as an important factor downregulating the NKG2D receptor mRNA and impairing the NK cell functions. The matter of interest is that TGF-β induces and IL-15 represses miR-1245 synthesis revealing a new connection in NK cell regulatory network (ref in Schenk et al. 2016).

Wang et al. (2012b) performed analysis of miRNomes resting and IFN-α-activated human peripheral blood NK cells and identified two miR-378 and miR-30e as suppressors of NK cell cytotoxicity by targeting granzyme B and perforin. Since it has been proven that in IFN-γ-primed macrophages the miR-3473b is strongly downregulated, which releases PTEN (phosphatase and tensin homolog) suppression and attenuates the microbicidal and tumoricidal activity of macrophages, it is possible that this can explain the molecular mechanisms for NK cell-derived IFN-γ-priming effect on macrophage activation (Wu et al. 2014). Detailed description of epigenetic mechanisms of NK cell regulations is given by Cichocki et al. (2013) and Sullivan et al. (2013). Separate issue concerns the role of miRs delivered by extravesicles as the intercellular communication in health and disease, particularly in cancer biology (Sato-Kuwabara et al. 2015).

5.6 Metabolic Reprogramming of Macrophages and NK Cells

Immune system cell development and interaction is supported by the engagement of various metabolic pathways. Recently developed trend, based on new advanced technologies, focuses on the investigation of relationship between metabolism (immunometabolism) and function of innate immunity cells including NK cells and macrophages (ref in Cheng et al. 2014; González Plaza et al. 2016). A comprehensive guide for immunologist, comprising the interplay between metabolic reprogramming including six major metabolic pathway and immune cell responses, is given by O'Neill et al. (2016). Cell reactivity and its consequence depend on the choice of particular metabolic pathways and their regulation by the substrate availability and involved signaling pathways (Pearce and Pearce 2013). Generally the short-lived immune cells depend on aerobic glycolysis, while the long-lived cells depend on the mitochondrial oxidative phosphorylation (OXPHOS) (ref in Loftus and Finlay 2016). Studies of IFN-γ production by primary murine NK cells reveal that the state of the rest and short-time activation *via* activating receptors mainly depend on glucose-dependent OXPHOS, while no such relation was found after cytokine pretreatment (IL-12+IL-18) and prolonged treatment with IL-15 (Keppel et al. 2015).

The essential role of mammalian target of rapamycin complex 1 (mTORC1) in control of cellular metabolism and immune responses has been intensively investigated. mTOR signaling pathway is critical for the IL-15-mediated NK cell activation during antiviral and antitumor responses (Marcais et al. 2014; Ali et al. 2015). The metabolic reprogramming of NK cells follows their activation. This is accompanied by the increase of mTORC1 activity that upregulates the rates of glucose uptake and glycolysis in vitro and in vivo. The direct limitation of glycolysis results in inhibition of NK cell IFN-γ production and cytolytic activity (Donnelly et al. 2014; Finlay 2015). The effect of short-time hypoxia and the hypoxia-inducible factor 1 (HIF-1) in positive synergistic interactions for genes encoding glycolytic pathway regulatory enzymes was observed. Beside the stimulation of glycolytic gene transcription, hypoxia also increased the migration and cytotoxicity of NK cells (Velásquez et al. 2016). On the other hand, hypoxia prevented the cytokine-mediated upregulation of NCRs and NKG2D but not CD16 in human peripheral blood NK cells (Balsamo et al. 2013). It also has been shown that cytolytic properties of NK cells against melanoma cells decrease under hypoxic conditions due to destabilization of immune synapse caused by lower level of gap-junctional connexin 43 (Tittarelli et al. 2015).

A shift from short-lived M1 macrophages to longer-lived M2 is accompanied by switching cellular metabolism from glycolysis to OXPHOS at the level of pyruvate kinase1 (Tan et al. 2015). Activated M1 macrophages are dependent on glycolysis with little participation of OXPHOS pathway, but integrity of mitochondria is required for cell survival (Pearce and Pearce 2013). Participation of mitochondrial energy generation and regulation of apoptosis in macrophage metabolic reprograming that promote and maintain their activation in bidirectional contacts

with other cells in the environment have been documented (El Kasmi and Stenmark 2015; Mills and O'Neill 2016).

Metabolic reprogramming of LPS-activated macrophages to glycolytic pathway with reduction of OXPHOS and mitochondrial enzymes activity is summarized by Kelly and O'Neill (2015) and Zhu et al. (2015). LPS signaling through TLR4 upregulates expression of iNOS and production of NO that inhibits OXPHOS components by nitrosylation of iron–sulfur proteins. LPS activation of mTOR affects the expression of HIF-1 and stimulation of glycolytic pathway also by direct interaction with target enzymes. Additionally, the impairment of mitochondrial beta-oxidation and dysregulation of citric acid cycle (CAC) enzymes is observed. That metabolic switch is typical for M1 type macrophages that release pro-inflammatory cytokines. Immune-suppressive M2 macrophages secrete IL-10 and TGF-β and reveal different metabolic pattern, depending on OXPHOS and CAC and active pathways including arginase-1 (Arg-1), β-oxidation of fatty acids, AMPK (AMP activated protein kinase), and PFKFB1 (6-fosfofructo-2-kinase/fructose-2,6-biphosphatase 1) (Kelly and O'Neill 2015; Izquierdo et al. 2015). The effect of INF-γ downregulating of mTORC1 and MNKs (kinases of MAPK-MNK axis), both triggering the regulator of translation initiation eIF4E in human macrophages, is associated with modulation of protein synthesis to potentiate macrophage activation, induction of inflammation, metabolic reprogramming, and autophagy (Su et al. 2015). Genome-wide analysis showed *s*elective expression of translationally regulated genes. Increased translation was observed for inflammatory cytokine and chemokine genes (IL-6, TNF, LTA, LTB, CXCL2, and CXCL3) and for genes associated with OXPHOS and mitochondrial pathways, while translation of the stress pathway genes was decreased (Su et al. 2015).

Recently the possible important role of cysteine cathepsins involved in activation of pro-granzymes into proteolytically active forms of serine proteases secreted through immune synapse and triggering the death targeted cells has been recognized as summarized by Perišić Nanut et al. (2014). Dynamic equilibrium among the exopeptidase cathepsin C expressed in NK cells and its endogenous inhibitor cystatin F found in endosomal/lysosomal vesicles determines processing the pro-granzymes and affects the NK cell cytotoxic action (Perišić Nanut et al. 2014).

It is evident that metabolic state of the organism and target cells has important impact over the function of NK and macrophages and that internal and/or external factors changing the metabolic homeostasis, e.g., obesity or virus infection, may create yet another level of innate immunity. It may also shape the patterns of intracellular interactions considering different NK cell/macrophage subsets.

5.7 Monocyte/Macrophage and NK Cell Interactions

Described mechanisms applied for decoding of environmental conditions and multiple levels of interacting signals provide an enormous plasticity in the programming of both NK cells and macrophages. Thus their interactions reveal the

plethora of combinations and patterns in realizing the common function of eliminating pathogens and transformed cells as well as participation in homeostasis of immune system. Both cell types can act complementary, synergistically, or antagonistically to maintain homeostasis in health and protect organism in disease. The recognition of their specific features in interaction with other components of innate and adaptive immune systems has been intensively studied, particularly in inflammation, in cancer development and progression, in transplantation immunology, and recently in autoimmune diseases.

From the very beginning of the investigations, it became clear that NK cells and monocyte/macrophage interaction is reciprocal (Weissman et al. 1986; Dalbeth et al. 2004). Two principal mechanisms of the cooperation between these two cell types have been investigated, direct stimulation by cell–cell contact and/or activation by variety of bioactive compounds, mainly cytokines.

5.7.1 Bacterial and Viral Infections

Macrophages and NK cells act in coordinated manner with feedback mechanism realized by secreted cytokines and cognate receptors depending on environmental conditions. Bacteria-induced activated macrophages release IL-15 and IL-12, the main factors stimulating activation and production of IFN-γ by NK cells that in turn further activates macrophages (Lapaque et al. 2009). This is accompanied by the release of chemokines attracting both types of cells to the site of infection (ref in Fehniger and Caligiuri 2001). The self-limitation of that cascade is possible by autocrine mechanism of TNF-α action in $CD56^{+}$ NK cells induced after prolonged stimulation by cytokines (Ross and Caligiuri 1997).

Beside direct activation of NK cells by prokaryotic and eukaryotic pathogens through TLRs, the accessory monocyte/macrophages play important role in that process. Many researches have proven that molecular pattern TLR ligands can induce NK cell cytotoxicity and/or cytokine production only in the presence of cocultured autologous activated monocytes or macrophages. This cooperation allows both cell populations adjust the immune response to the variety of physiological and pathologic conditions in various microenvironments. Numerous studies showed that depending on monocyte/macrophage stimulatory factors, as well as on NK cell subset, either cytotoxicity or cytokine production takes place. $CD56^{bright}$ NK cells after stimulation by monokines produce significantly greater levels of cytokines including INF-γ, TNF-β, GM-CSF, IL-10, and IL-13 than $CD56^{dim}$ NK cells. Moreover, the cytokine repertoire depends on specificity of monokines secreted by cells present in environment, e.g., IL-15 induces type 2 cytokine production by $CD56^{bright}$ NK cells. That immunoregulatory cytokines are involved in tuning the innate and adaptive immune response what corresponds to the regulatory role of that particular NK cell subset (Cooper et al. 2001a).

An early stage (starting from 1990s) of research concerning the NK and monocyte/macrophage interactions in health and under pathogenic conditions in mice and humans is summarized by Malhotra and Shanker (2011) and Michel et al. (2012). These authors described potential role of activated monocyte/macrophage in upregulation of the expression of NK cell-activating ligands MHC1 chain-related molecule A and B (MICA and MICB) and secretion of cytokines, including pro-inflammatory IL-12, IL-18, TNF-α, type I IFN, and chemokine CCR7. Required cell–cell interactions include important role of NKG2D, NKp30, NKp44, NKp80, 2B4, and CD69 receptors on human NK cells and specific macrophage ligands expressed in the manner dependent on how macrophages have been primed (bacteria, viruses, fungi, and parasites). Bacteria- and virus-stimulated monocyte/macrophage and Kupffer cell (KC) interaction with NK cells affecting IFN-γ production and CD69 expression was summarized by Souza-Fonseca-Guimaraes et al. (2012).

The NK cell expansion or differentiation is not affected by IFN-γ in endocrine manner, but together with secreted TNF-α is a part of NK cytolytic function through ICAM-1 expression in target cells promoting immune synapse formation (Wang et al. 2012a). IFN-γ is critical for macrophage activation. The pleiotropic action of IFN-γ in macrophages comprises stimulation of microbial killing and inflammatory reaction activation via TLRs, the translational regulation, metabolic reprograming, and autophagy (Hu and Ivashkiv 2009; Su et al. 2015). It has been proven that in IFN-γ-primed macrophages, the miR-3473b is strongly downregulated resulting in the release of PTEN suppression and attenuation of their microbicidal and tumoricidal activity. It is possible that this can explain the molecular mechanisms of NK cell-derived IFN-γ-priming effect on macrophage activation (Wu et al. 2014).

Interesting is the effect of IL-10 on both NK cells and macrophages and their interaction. In macrophages, the IL-10 induces elevated expression of the NKG2D ligands MICA and MICB, as well as UL16-binding proteins (ULBP)—ULBP-1, ULBP-2, and ULBP-3. Thus, increased expression of NKG2D receptors on NK cells and its ligands on macrophages by IL-10 result in increased lysis of autologous macrophages by NK cells, as it is observed under inflammatory conditions (Schulz et al. 2010).

Pointed out should be the differences due to tissue origin of cell populations, particularly the influence of tissue environment on macrophage polarization and its effects on NK function (Bellora et al. 2010; Romo et al. 2011). Macrophages stimulated by microbial products polarize toward M1 phenotype that leads to NK cell activation. Activated NK cells secrete macrophage-activating factors such as GM-CSF, TNF-α, MIP1αβ (human macrophage inflammatory protein 1 alpha and beta), and IFN-γ which, in turn, further activate macrophages and promote their polarization to M1. Resting and M2 macrophages are eliminated due to their low MHC1 expression, while overstimulated macrophages are destroyed via increased expression of NKG2D stress-inducible MHC class I-like ligands on macrophages (Nedvetzki et al. 2007). In contrast to activated macrophages, the resting microglia (resident brain macrophages) are killed by NK cells through NKG2D and NKp46

recognition that might restrict immune responses within the human central nervous system (Lünemann et al. 2008).

The mechanism of that process was revealed by Nedvetzki et al. (2007) who recognized and documented the two functionally distinct types of macrophage–NK cell immune synapses: non-lytic, responsible for proliferation and cytokine secretion, and cytolytic, involved in lysis of targeted cells. The matter of interest is that in lytic synapses between NK cells and LPS-activated macrophages, the accumulation of NK cell-derived F-actin was observed, while at non-cytolytic synapses the macrophage-derived F-actin was located. Also evidences were collected considering the importance of NKG2D, DAP10, and CD3ζ but not 2B4 accumulation at the NK cell lytic synapse with LPS-activated macrophages, confirming the role of former three molecules in triggering NK cytotoxicity, whereas 2B4 activation of NK cells induced their proliferation, but not lytic properties. Lünemann et al. (2008) showed that IL-2-activated NK cells in cocultures effectively destroyed resting allogeneic and autologous microglia by direct cytotoxic mechanisms dependent on NKG2D and NKp46 and synapse formation followed by perforin polarization to the interface and stimulation of IFN-γ production. In contrast to LPS-activated macrophages, the binding of LPS to microglia TLR protected the cells against NK toxicity, which was due to the upregulation of MHC class I surface expression and activation of inhibitory receptors.

Virus infections remarkably affect NK cell homeostasis, phenotype, and functions, during acute and chronic viral disorders inducing amplification of antiviral immune responses or developing mechanisms to evade NK cell-mediated defense reactions (Lugli et al. 2014). The induction of unconventional $CD56^{neg}$ NK cells was observed with impaired effector functions. NK cell and macrophage survey against viral infections occurs due to secretion of macrophage-recruiting chemokines CCL3/MIP1α, CCL4/MIP1β CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), activating cytokine IFN-γ, and GM-CSF. Generally, the virus-infected human macrophages produce IFN-α/IFN-β that is a potent stimulator of IFN-γ secretion by NK cells depending on cell–cell contact. Expression of MICB gene in infected macrophages and enhanced synthesis of IL-12Rβ2 and IL-18R and T-bet mRNA in NK cells, both lymphoblasts and isolated from PB, was also observed (Sirén et al. 2004). Arenaviruses-infected macrophages induce NK cell proliferation and cytotoxic activity but not IFN-γ production. This was accompanied by upregulation of CD69, NKp30, and NKp44 and the downregulation of CXCR3, but no virus suppression was seen (Russier et al. 2012). Marras et al. (2014) observed interindividual differences in expression and inducibility of NK cell NCR NKp46 and NKp30 kinetics in patients with HIV and HCV chronic infections that may explain their divergent disease courses. Comprehensive review of the role of NK cell in controlling human virus diseases and involved receptors and ligands is given by Jost and Altfeld (2013) and NK cell cooperation with accessory cells by Wang et al. (2013) and Mandal and Viswanathan (2015).

It has to be remembered that activation of NK response to different pathogens, including viruses, bacteria, and parasites, often requires and depends on the

cooperation with various other accessory cells and their cytokines as well as with stroma cells in addition to monocytes/macrophages (Newman and Riley 2007).

5.8 Liver as Innate Immune Organ

Particular role of the liver as main site of xenobiotics biotransformation maintaining the metabolic and immunologic homeostasis at the tissue and at systemic level is summarized by Robinson et al. (2016). Production of many soluble serum proteins maintains the homeostasis of blood clotting and complement systems, whereas under pathogen invasion the liver supports increased amount of acute-phase compounds participating in defense. That spectrum of functions is realized by wide cell repertoire including hepatocytes, epithelial cells, Kupffer cells (KC), monocyte/macrophages, and diverse populations of liver lymphocytes. Liver-resident macrophages (KC), localized at high number within the lumen of the liver sinusoids, are well characterized as cells that participate in the detoxification and removal of blood endogenous and environmental substances (Bilzer et al. 2006).

Due to pleiotropic liver functions, comprising metabolic and remodeling processes, the hepatic immune system is under continuous environmental exposure to dietary and microbial products. The maintenance of liver homeostasis requires appropriate control over inflammatory response induced by pathogens or tissue damage. Disruption of homeostasis results in chronic infections, autoimmunity, and malignant transformation, which progress to liver failure (Eckert et al. 2015). Various pleiotropic functions of the liver are realized due to very specific immune mechanisms including interaction of immune and non-hematopoietic cell populations (Crispe 2009).

In the recently published article, Peng et al. (2016) pointed out the predominant role of the liver in innate immunity with particular attention to the distinct NK cell subsets (NK cells, NKT, and γδ-T cells) that amount to 65% of liver lymphocyte population and liver macrophage phenotype (Kupffer cells, KC) with specific surface marker expression, cytokine profiles, and cytotoxic potential (Doherty and O'Farrelly 2000; Gallina et al. 2008; Gao et al. 2009a; Robinson et al. 2016). Hepatic NK cells reside in distinctive, thin-walled sinusoids of the liver, where Kupffer cells are also located. That creates conditions for the very specific interaction between those cell populations (Seki et al. 2000; Hudspeth et al. 2016).

Characteristics of two liver macrophage populations are summarized by Ju and Tacke (2016). Liver-resident macrophages, Kupffer cells (KC), originate from the local stem cells delivered during embryogenesis and infiltrated monocyte-derived macrophages originated from BM. Both populations display distinct phenotypes and functions, first being mostly tolerogenic and second the immunogenic. Circulating monocytes include subsets of immunogenic $CD14^{++}CD16^{-}CD14^{high}$ monocytes gathering fast in the sites of inflammation and differentiating into inflammatory macrophages and $CD14^{+}CD16^{++}CD14^{dim}CD16^{++}$ patrolling

monocytes associated with wound healing and the resolution of inflammation in damaged tissues (Liaskou et al. 2013; Stansfield and Ingram 2015).

KC and NK cells play a central role in liver regeneration by promoting hepatocyte proliferation via release of IL-6 and TNF-α, while the liver-resident NK cells inhibit hepatocyte proliferation through IFN-γ production, which induces hepatocyte cell cycle arrest (Sun and Gao 2004; Tan et al. 2016).

Activated KC primarily release CCL2 and pro-inflammatory cytokines such as IL-6 and IL-18, but also release IL-10 that induces expression of NKG2A in liver-resident NK cells (Krueger et al. 2011). The liver functions as an immunotolerogenic organ, that is also explained by the release of IL-10, which is stimulated via TLR by lipoteichoic acid (LTA) and LPS derived from commensal bacteria in the intestine. IL-10 induces hyporesponsivity of NK cells to IL-12 and IL-18 (Tu et al. 2008; Shi et al. 2011).

The phenomenon of the liver's ability to protect from gut-derived pathogens with simultaneous tolerance to food and commensal organism-derived antigens has been investigated. The main players in that mechanism are Kupffer cells and liver NK cells. Their direct interaction makes possible simultaneous transfer from KC to NK cells through TLR of activating (IL-18) and inhibitory (IL-10) signals, with modulatory action of MDF88 (myeloid differentiation factor 88) inducing IL-10 and IRF3 (IFN-β regulatory factor 3) decreasing the IL-10 production. That may cause the subversion of immune response against viruses (Tu et al. 2008).

5.9 Hypersensitivity Reaction-Dependent Diseases

5.9.1 Autoimmune Disorders

The described NK cells' new features and complicated, multilevel regulatory mechanisms resulting from cooperation with autologous cells and external stimuli point out to their critical role in the organ-specific and systemic hypersensitivity-dependent autoimmune diseases, particularly in the view of the viral infections involved in pathogenesis of certain autoimmune disorders. Autoimmunity belongs to adaptive hypersensitivity reactions and is triggered by dysregulation of various T-cell and B-cell subset activity against self-components of host organisms. NK cells interacting directly with normal self-cells and/or extracellular matrix components and secreting cytokines play a role in maintaining or disrupting tissue homeostasis and protect or initiate autoimmunity in the cooperation with adaptive immunity components (Poggi and Zocchi 2014).

The regulatory NK cells are enriched in immunotolerant organs and play important role in immune system homeostasis acting as controller or inducer of tissue-specific and systemic autoimmune reactions (Sun et al. 2013). The investigations of the potential role of NK cells in autoimmune diseases have been restricted, due to

the limited human clinical materials, animal models, and in vitro studies (Tian et al. 2012).

The early investigations reported quantitative and qualitative differences in NK cell properties generally describing a reduction in the number of circulating NK cells and decrease in their cytotoxicity. Detrimental action of NK cells in autoimmune diseases is due to direct activation of their natural cytotoxicity receptors (NCRs) and by ADCC through CD16 receptor and indirectly, through stimulation of maturation and activation of APC, including macrophages, by releasing IFN-γ. Protective role of NK cells results from controlling the environmental factors such as viral infections and macrophage activity by secreted IL-10, IL-5, and IL-13 (Flodström-Tullberg et al. 2009; Schleinitz et al. 2010).

The participation of NK cells in initiation and progression of autoimmune responses has been described over the years. Early observations considered the impaired functions and decreased number of circulating NK cells in many autoimmune disorders including multiple sclerosis (MS), systemic lupus erythematosus (SLE), hemophagocytic lymphohistiocytosis (HLH), rheumatic diseases, systemic juvenile rheumatoid arthritis (sJRA), diabetes, and psoriasis. Further studies identified distinct subsets of NK cells, their properties, and genetic analysis (ref in Lünemann et al. 2009; Malhotra and Shanker 2011). Both protective and destructive influence of NK cells was observed in multiple sclerosis (Kaur et al. 2013; Høglund and Maghazachi 2014). The specific phenotype of NK17/NK1 cells was found in cerebrospinal fluid of patients with MS. These cells express $CD56^{+}$ and $CCR4^{+}$ and produce IL-17 and IFN-γ and may be involved in pathogenesis of disease (Pandya et al. 2011). The importance of macrophage–NK interactions has been found in many rheumatic diseases including systemic juvenile rheumatoid arthritis (sJRA) characterized by severely decreased NK lytic activity and an absence of circulating $CD56^{bright}$ NK cells together with macrophage activation syndrome (MAS) and secretion of high levels of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) responsible for tissue damage and clinical symptoms (Villanueva et al. 2005).

The role of NK cells in development of type I insulin-dependent diabetes (T1D) characterized by pancreatic beta cell (β cell) destruction has been investigated and confirmed in animal models and humans (ref in Enk and Mandelboim 2014). Lower expression of NCL subtype activating receptors NKp46 and NKp30 on NK cells in PB of patients was observed during long-course disease and also NKG2D in newly diagnosed patients, while the higher level of activating KIR genes was noticed in NK of T1D patients, as compared to matched controls. The presence of unknown ligand for NKp46 on β cells of normal and T1D mice and humans has been described. NKp46 ligand is mainly located in insulin granules and after stimulation by glucose undergoes translocation to the cell membrane (Gur et al. 2013). It has been suggested that localization of NK cells in close proximity to β cells is responsible for NK destructive function and that it may affect the susceptibility of certain individuals to T1D.

Growing evidence support the role of immune system in type 2 insulin-non-dependent diabetes (T2D) characterized by the β cell dysfunction and insulin

resistance. T2D is promoted by obesity-induced insulin resistance and is connected to chronic inflammation of abdominal adipose tissue. The presence of specific phenotype of adipose tissue-resident NK cells and accumulation of inflammatory macrophages play a critical role in development of that disorder. Mouse model studies revealed that obesity stimulates expression of adipocyte ligands for NK cell-activating receptors promoting their proliferation and INF-γ production that induce macrophage accumulation and polarization toward M1 inflammatory type (Wensveen et al. 2015; Lee et al. 2016). T2D patients have decreased PB number of NK cells expressing NKG2D and NKp46 receptors and lower cytotoxicity in peripheral blood that may explain their high susceptibility to infections (Berrou et al. 2013).

Breaking tolerogenic state of liver immune system induces liver autoimmune diseases such as primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), and primary sclerosing cholangitis (PSC) and is accompanied by the unique pattern of NK cell phenotypes. The various clinical courses of diseases can be regulated by possible interaction among other immune cells including monocytes and KC (Hudspeth et al. 2013a, b; Jiao and Wang 2016).

Comprehensive reviews of a potential immune-regulatory role of NK cells in autoimmune disorders in humans are given by Fogel et al. (2013) and Folci (2014).

Considering the variability of autoimmune disorders, particularly including inflammatory immune diseases such as allergic asthma, the precise role of specific subsets of NK cells and their regulatory mechanisms as well as cooperation with cognate monocyte/macrophage phenotypes need to be further determined.

5.9.2 Contact Hypersensitivity Reactions

Investigations of NK cell participation in hypersensitivity reactions begun from the observations in murine models. O'Leary et al. (2006) showed that contact hypersensitivity reaction to haptens in mice can be induced independently of T and B cells and that specific memory-like NK cells are found in the liver of sensitized animals.

Classical T-cell-mediated immunity to sensitizing haptens, mostly low molecular xenobiotics, toxins, and metal elements, manifests as a contact dermatitis reaction (CD) and is classified as type IV hypersensitivity reaction (CHR) or delayed-type hypersensitivity reaction (DTH). Haptens became immunogenic after binding to host proteins. Following processing by antigen-presenting cells, the specific Th memory cell population is induced. Second exposure is followed by the inflammatory reaction and reactive oxygen species (ROS) production, NLRP3 inflammasome activation, and IL-1β release (Martin et al. 2011). Involved are helper Th1 subtype and cytotoxic T lymphocytes, both releasing IFN-γ, with participation of T17 lymphocytes secreting IL-17 (Chipinda et al. 2011). Involvement of other innate-like types of lymphocytes including γδ-T cells, invariant NKT

cells, and specific B1 lymphocytes is also documented (Askenase 2001; Martin et al. 2011).

Participation of macrophages and NK cells in contact hypersensitivity reactions (CHR) have been studied in vitro and in vivo in murine models including different lymphocyte-deficient mice, i.e., *CD3ε*$^{-/-}$ (T-cell-deficient mice) and *Rag1*$^{-/-}$ (T- and B-cell-deficient mice), SCID^{beige} mice (have nonfunctional NK cells) (Majewska-Szczepanik et al. 2013). These investigations have shown requirements for IFN-γ production during an early onset of NK cell-dependent contact sensitivity and for IL-12 and IFN-α for liver-delivered antigen-primed mature NK cells. It is important to notice that Rouzaire et al. (2012) who compared CHR reactions mediated by NK cells and T cells in mice have found lack of cellular infiltrate and cytotoxic markers in NK cell-mediated response as well as no effect of repeated antigen challenge.

In human, majority of haptens induce allergic contact dermatitis (ACD) reactions in the skin. Investigations of the ACD in human confirmed the presence of NK cells in inflammatory infiltrate together with various subsets of T lymphocytes, dendritic cells, and NKT cells. In the skin of nickel-induced ACD patients, the subset of CD56^{high}CD16^{-} CD62L^{-} NK cell that expressed NKG2A, the high level of NKG2D, NKp44, and NKp46 activating receptors and CXCR3, CCR5, and CCR6 chemokine receptors, released IFN-γ and TNF-α but not IL-17 or IL-22, and induced keratinocyte apoptosis, however in a hapten-non-dependent manner, was identified by Carbone et al. (2010). The effector response in vitro of NK cells from nickel-allergic donors depends on the presence of IL-2, which pointed out the importance of interaction with other immune cells including Th1 lymphocytes (Carbone et al. 2010).

The role of NK cells in elicitation of CHR reaction is still under investigations and so far its participation is only known for certain NK subset residing in the liver. Min-Oo et al. (2013) described the liver-restricted memory cells stimulated by hapten or specific antigen in conjunction with CXCL16 molecule. Similar subset of NK memory cells was found only in the skin (Sojka et al. 2014).

Extensive studies confirmed presence of similar human memory-like NK cell types. The cytokine pre-activation of CD56 NK cells in vivo with cytokine combinations including IL-12, IL-15, and IL-18 enhanced permanently IFN-γ production after restimulation, followed by the expression of CD94, NKG2A, NKG2C, and CD69 as well as CD25 and high-affinity of IL-2Rαβγ receptor, and a lack of CD57 and KIRs (Romee et al. 2012; Leong et al. 2014).

Peng et al. (2013) described the phenotype and functional properties of memory-like type liver-resident NK cells as different subpopulation. In mice the NK cells having the memory of hapten-specific contact hypersensitivity reaction originated from hepatic hematopoietic progenitor cells (HPC) were CD49a^{+}DX5^{-}. This population is located exclusively in the liver sinusoid compartments and expresses genes associated with negative regulatory action and activation of immune tolerance at much higher level than CD49a^{-}DX5^{+} NK cell population, circulating and residing in other peripheral organs. Authors suggest that hapten-induced

sensitization of NK cells may occur in the liver but not in the spleen. Moreover, the $CD49a^{-}DX5^{+}$ NK cells were found at the hapten-induced skin lesions.

The liver-resident memory NK cells have probably multisource origin such as the adult bone marrow hematopoietic progenitor cells (HPC) or fetal liver HPC (Jiang et al. 2013). Considering the fact that the liver is the main site of biotransformation of xenobiotics carried to that organ by the blood stream, it seems reasonable that antigen recognition by NK cells happens primarily in the liver, even though response is observed at challenge site located at periphery (Peng et al. 2013). Possibly the resident $CD49a^{+}DX5$ NK cells with memory potential are activated and proliferate in the liver. Second challenge with the same hapten that may happen at distal site, followed by transfer to the liver by blood or by antigen-presenting cells, should cause induction of their effector functions and departure from the liver. Other explanation includes sensitization of NK memory-like cells in peripheral lymph nodes by antigen-presenting cells, followed by circulation in blood and localization in the liver directed by specific chemokine receptor CXCR6 (Paust et al. 2010). After second challenge resident NK cells are recruited back to circulation and the site of reaction (Paust and von Andrian 2011).

The molecular mechanisms, regulatory aspects, and epidemiology of contact chemical hapten-specific skin reaction are reviewed by Peiser et al. (2012) and Erkes and Selvan (2014). Major concepts of hapten-induced contact dermatitis include three-phase reaction mechanisms: sensitization phase, early elicitation phase following the second hapten challenge, and late elicitation phase. Macrophages participate in second and third phase, and hepatic CXCR6+ NK cell trafficking through circulation was documented in the third phase. At all above stages, the cooperation of the other immune cells present in the epidermis (Langerhans cells, keratinocytes) and dermis (mast cells, dDC, and macrophages) and their cytokines, mainly IL-1β, IL-18, and TNF-α, are required for liver NK activation.

In the search for hapten recognition mechanisms of NK cells, Grandclément et al. (2016) followed the observation that certain hapten-induced CHR were accompanied by the rapid cellular Ca2+ ion influx through specific plasma membrane channels. Proposed mechanisms include store-operated calcium entry (SOCE) pathway requiring activation of plasma membrane calcium release-activated channel (CRAC) proteins ORAI by Ca2+ ions released from endoplasmic reticulum stores through calcium sensor stromal interaction molecule (STIM) 1 and 2 and participation of transient receptor potential (TRP) channels, particularly TRPC3 channel, as well (Feske et al. 2015).

5.9.3 Atopic Allergy

Atopic allergic diseases, including atopic dermatitis (AD), allergic rhinitis (AR), food allergy, anaphylaxis, and asthma, are type I hypersensitivity reactions dependent on overexpression of immunoglobulin E (IgE) and on mechanisms involving

innate and adaptive immunity (Oettgen 2016). In sensitization phase allergen-activated B cells produce immunoglobulin E (IgE), and effector cells, IgE-coated mast cells and basophils, activated eosinophils, and Th2 cells and their cytokines such as IL-4, IL-5, and IL-13 are involved (Stone et al. 2010; MacGlashan 2012). After second allergen exposure atopic allergy reaction is characterized by two-phase mechanisms of an "immediate" (15 min) and "late" (up to 48 h) response, the latter including macrophage infiltration (Galli et al. 2008). The involvement of NK cells is under investigation; however, it is far from being fully understood.

The early investigations concerning the NK cells involvement in atopic dermatitis revealed the presence of NK2 subset in PB of patients (ref in von Bubnoff et al. 2010). Further studies indicated differences in numbers, surface receptors, secreted cytokine pattern, and function of NK cells in atopic allergy. AD patients have reduced number of $CD16^{+}CD56^{+}$ NK cells in PB, and these cells released higher amounts of interleukin IL-4, IL-5, IL-13, and IFN-γ after in vitro stimulation (Aktas et al. 2005), while in PB of patients with AR, conflicting results have been published (higher number and increase cytotoxicity of NK cells versus lower number of CD56++CD16+/− and lower IFN-γ secretion) (Mesdaghi et al. 2010; Scordamaglia et al. 2008). NK cells from AD patients undergo apoptosis upon stimulation in the presence of monocytes parallel with the impairment of TNF-α and IFN-γ but no IL-4 production. That phenomenon was not due to IL-10 secretion by monocytes but was depended on direct cell–cell contact (Katsuta et al. 2006). The regulatory NK cells secreting IL-10 and TGF-β suppress IgE production, while NK2 subset produces IL-5 and IL-13 and stimulates IgE production (Deniz et al. 2013). The matter of interest is stimulation of chemokine production by histamine in human NK cells through histamine H4 receptor (H4R) (Mommert et al. 2015).

Experiments in vitro with human NK isolated from PB and with NK cell line and in vivo in mice revealed that significant amount of pro-inflammatory cytokine IL-13 was produced upon stimulation by IL-2 in the absence of IFN-γ. It has been suggested that the balance between NK cell subsets producing IFN-γ or secreting IL-13 and other immune cells producing Th2 type cytokines may affect the type of immune response as either humoral or cytotoxic reactions (Hoshino et al. 1999). IL-13 belongs to activating cytokines for macrophages, and its participation together with IL-4 in allergic lung and skin inflammation was described. Comprehensive review of cytokine signaling in allergic diseases is given by Cara et al. (2012).

5.10 Cancer

It is well recognized that developing cancer creates a unique microenvironment in itself and in surrounding tissues. The macrophages and NK cells recruited to the tumor site can undertake the destructive action, but they can also become subject of immunosuppression mechanisms. The numerous reviews summarize the current

knowledge concerning the development and function of tumor-associated macrophages (TAM) that promote cancer progression and metastasis and their inhibition of NK cell function.

As a result of interactions between tumor components, stromal cells, and resident or recruited immune cells, the developing solid tumors realize chronic inflammatory response that allows them to escape the host defense. Tumor microenvironment (TME) represents specific conditions created by hypoxia and tumor-derived growth factors, immunosuppressive cytokines, and other soluble factors. That process is also strongly supported by the cells present in TME such as macrophages, myeloid-derived suppressor cells, Treg cells, and fibroblasts. Under such conditions the tumor-associated/tumor-infiltrating macrophages and NK cells polarize toward specific phenotypes that realize cancer destruction or become immunosppressive (Krneta et al. 2013; Vitale et al. 2014).

5.10.1 Tumor-Associated/Tumor-Infiltrating Macrophages

Tumor-associated macrophages (TAMs) are abundantly present in the stroma of solid tumors and modulate several important biological cancer processes, such as neoangiogenesis, cancer cell proliferation and invasion, and suppression of adaptive immune responses. Monocyte recruitment and macrophage survival and proliferation depend on tumor-derived chemotactic factor (MCP-1 or CCL2), cytokines, and growth factors such as VEGF and M-CSF. Tumor-associated macrophages can exert dual function promoting or destabilizing tumor development and progression in manner depended on TME signals (Lewis and Pollard 2006). M1 macrophages realize antitumor response by secretion of IL-12 and TNF-α, while M2 phenotype promotes growth, angiogenesis, and extracellular matrix deposition promoting metastasis by secreting IL-10 and IL-1 receptor antagonist (IL-1 Ra) and IL-1 decoy receptor (Mantovani et al. 2008; Sica and Bronte 2007; Mantovani and Sica 2010). Tumor-derived cytokines such as IL-4, IL-10, IL-13, and M-CSF convert TAM into M2 subtype resulting in immunosuppressive activity (Mantovani et al. 2002). Such M2 subtype TAMs support neoplastic transformation, progression, and subsequent metastatic cascade. Studies indicate the role of TAM-expressed C-type lectin receptors in polarization toward an immune-suppressive profile with increased IL-10, lack of IL-12, and decreased CCL3 (attracting Th1 lymphocytes) expression. TAMs isolated from human ovarian carcinoma express high level of CLRs including mannose receptor (MR) and dectin-1-binding tumoral mucins (CA125 and TAG-72) (Allavena et al. 2010). Recently the role of dectin-1 in recognition of specific structures (*N*-glycan, lipids, proteins) on some cancer cells and stimulation of tumoricidal activity of NK cells has been described (Chiba et al. 2014). It has to be noticed that TAMs isolated from certain tumors (RCC, renal cell carcinoma) present a hybrid phenotype population sharing both M1 and M2 properties. They secrete pro-inflammatory cytokines

TNF-α, IL-1β, IL-6, and CCL2. This together with production of VEGF stimulates expression of metalloproteinases (MMPs) by tumor cells and angiogenesis, respectively (Kovaleva et al. 2016). Using cocultures of mouse cells, Krneta et al. (2016) documented that M2 macrophages derived from BM and obtained from PB strongly inhibit NK cell activity and cytotoxicity against tumor cells in cell-cell-dependent manner involving the TGF-β.

5.10.2 Tumor-Infiltrating/Tumor-Associated NK Cells

Mechanism by which the NK cells may be recruited to solid tumors has been recently revealed by Parodi et al. (2015) who demonstrated that NK cells upon interaction with melanoma cells release the chemotactic form of high mobility group box-1 (HMGB1), attracting activated NK cells in paracrine manner. This is accompanied by engagement of different activating NK cell receptors and changes in expression of cytoskeletal proteins promoting cell motility. Increased expression of RAGE receptors is involved in HMGB1-induced NK cell chemotaxis.

The role of NK cells in progression and angiogenesis of various human cancers has been reviewed by Stojanovic et al. (2013) and Bruno et al. (2014). Tumor-associated (TANKs) and particularly tumor-infiltrating (TINKs) NK cells showed impairment cytotoxicity with a drastic reduction of receptor expression including NKp30, NKG2D, DNAM-1, and CD16. The major phenotype of TINKs identified in human cancers belongs to the $CD56^{bright}perforin^{low}$ subset. TINKs and TANKs produce proangiogenic factors such as VEGF, PDGF (placenta-derived growth factor), and IL-8 (ref in Bruno et al. 2014). The proangiogenic function of NK cells was confirmed in inflammatory angiogenesis model in mice. The in vivo depletion of ocular NK cells reduces corneal angiogenesis and reduces macrophage infiltration into the cornea (Lee et al. 2014). Vacca et al. (2013) documented that NK cells isolated from pleural effusion (PEs) of patients with different types of cancer belong to $CD56^{bright}$ phenotype. Under stimulation with IL-2, they expressed normal levels of both activating and inhibitory receptors and reveal a high cytolytic activity against allogeneic and autologous tumor cells by releasing IFN-γ and TNF-α. The cytolytic activity was mediated mainly by NKG2D and NKp30 and partially by NKp46 and DNAM-1. It is obvious that anergy of TINKs/TANKs is due to localization in TME.

The matter of interest is that various types of human stem cells including human embryonic (ESCs), mesenchymal (MSCs), adult (ASCs), induced (ISCs), and various cancer type (CSCs) are more susceptible to NK cell-mediated cytotoxicity than their more differentiated forms. The destruction of cancer stem cells by NK is disrupted in coculture with monocytes. In the presence of monocytes, in parallel with decrease of NK cytotoxicity against cancer cells, there is stimulation of IFN-γ production (so-called "split anergy" effect) (ref in Jewett et al. 2012). The further studies of Tseng et al. (2015) using an LPS-stimulated human monocytes and NK cells tried to explain this phenomenon. They showed that LPS potentiated the loss

of human NK cell cytotoxicity while increasing IFN-γ secretion in the presence of monocytes. Similar results were obtained with monocytes isolated from myeloid-specific COX-2 knockout mice.

Interesting observation was made recently by Bui et al. (2015) who revealed that probiotic bacteria AJ2 (a combination of eight Gram-positive bacterial strains) induce significant split anergy in activated NK cells promoting differentiation of tumor cells and resistance to NK cell-mediated cytotoxicity. In the presence of probiotic bacteria, monocytes secreting IL-10 synergize with NK cells in that process acting on differentiation of cancer stem cells, augmenting resistance to NK cell cytotoxicity, and inhibition of cytokine release.

5.11 Conclusions

Understanding the mechanisms of NK cell activation, development/differentiation into functional subsets, and cooperation with their innate monocyte/macrophage partners has critical impact in the development of successful NK cell-based therapies. The NK cells, together with macrophages, emerge as major players in bacterial and viral infections; hypersensitivity reaction-based diseases, including autoimmunity disorders; and cancer. The evolving target is to develop the combinatorial therapeutic regimens comprising traditional chemotherapy, radiotherapy, and surgery with modulation of cytotoxicity and/or immune system controlling properties of NK cells and macrophages ex vivo and in vivo.

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Chapter 6
Macrophages in Invertebrates: From Insects and Crustaceans to Marine Bivalves

Prasad Abnave, Xavier Muracciole, and Eric Ghigo

Abstract Macrophages are critical components of the antimicrobial response. The recent explosion of knowledge on the evolutionary, genetic, and biochemical aspects of the interaction between macrophages and microbes has renewed scientific interest in macrophages. The conservation of immune components or mechanisms between organisms during the evolutionary process allows us to elucidate antimicrobial mechanisms or discover new immune functions through the study of basal-branching organisms, such as invertebrates. As a result, immunity in non-vertebrates has attracted the attention of researchers in the last few decades. In this review, we summarize what is presently known about macrophage-like cells in various invertebrate species.

Abbreviations

PAMPs	Pathogen-associated molecular patterns
PGRP	Peptidoglycan recognition protein

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PRR	Pattern recognition receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TEP	Thioester-containing protein

6.1 Introduction

Macrophages are professional phagocytic cells that are key components of the innate and adaptive immune system. They are known to help clear invading pathogens as well as potentially harmful altered cells and cell debris. In addition, macrophages are Trojan horses for several bacterial pathogens (Weiss and Schaible 2015). Apart from their antimicrobial role, macrophages also play a critical role in various diseases (e.g., cancer) and physiological processes (e.g., aging and regeneration) (Wynn and Vannella 2016). Their distribution in the human body is also diverse. They can be found in various locations in the human body such as in the liver, lymphoid organs, lungs, bone marrow, skin, and gastrointestinal tract (Epelman et al. 2014). Given their presence in various organs and their role in biologically significant processes, macrophages have become one of the most important immune cells under investigation. The key research interest is to understand the process of phagocytosis—an intracellular signaling for effective pathogen clearance, different antimicrobial mechanisms, and discrimination of self versus nonself (Kaufmann and Dorhoi 2016).

Several vertebrate and invertebrate animal models have been used to investigate the phagocytic process. In vertebrates, besides macrophages, several other types of immune cells such as neutrophils, monocytes, mast cells, and dendritic cells are known to have a phagocytic function (Gordon 2016). In invertebrates, these distinct classes of immune cells are not well defined, and all phagocytic cells are described under the generic term of hemocytes or phagocytic hemocytes (Buchmann 2014). Invertebrate hemocytes share several functional similarities with phagocytic cells in vertebrates (Browne et al. 2013). Because of the absence of adaptive immunity, genetic tractability, and amenity to high-throughput screenings as well as the conservation of the innate immune function during the evolution of species (Buchmann 2014) (Fig. 6.1), invertebrates have proven to be powerful models to investigate the conserved functions of phagocytes. A simple analysis in PubMed reveals the scientific community's interest in hemocytes. Indeed, a search based on the keyword "hemocytes" shows that the number of papers published annually tripled (300% increase) between 2000 and 2015, increasing from 107 to 338 papers per year (Fig. 6.2). In 2016, at the time of this chapter's preparation, 269 papers had already been published (Fig. 6.2). Here, we will not discuss phagocytes in vertebrate models, because they have been extensively reviewed in the literature. We instead decided to focus on invertebrate model organisms, such as insects, crustaceans, and bivalves. Many are called "exotic models" in research programs, because they are not the common model organisms used in biological research.

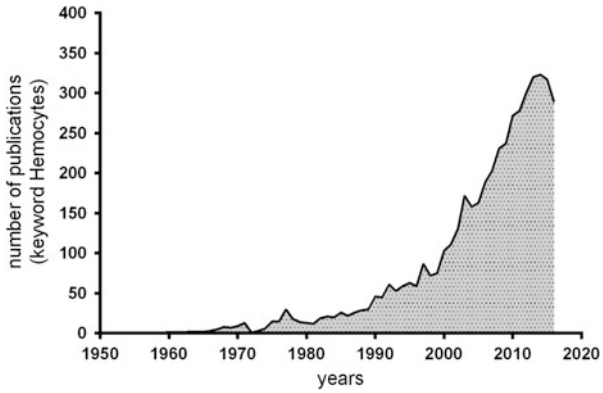

Fig. 6.1 Number of publications related to the keyword "hemocytes" (PubMed database)

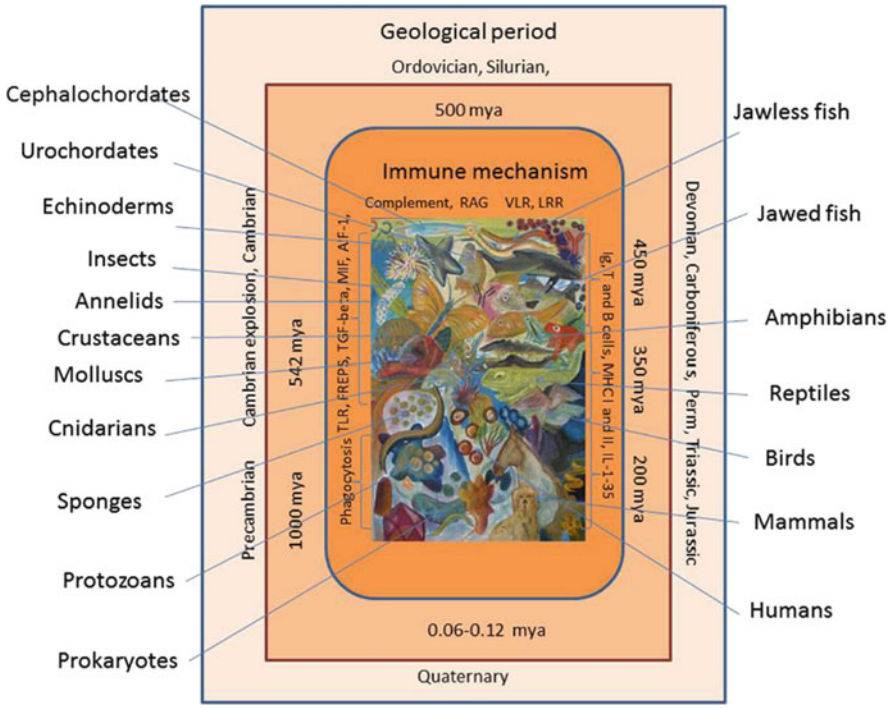

Fig. 6.2 Evolution of the immune system. The evolution of immune cells and molecules from early invertebrates to vertebrates is shown. Geological periods and time periods (million years ago, mya) are shown with extant representatives of animal groups appearing at different times during evolution. The illustration is taken from an open-access article, distributed under the terms of the *Creative Commons Attribution License* (*CC BY*). Its use, distribution, or reproduction in other forums is permitted, provided that the original author(s) or licensor is credited and that the original publication in this journal is cited, in accordance with accepted academic practice (Buchmann K (2014) Evolution of innate immunity: clues from invertebrates via fish to mammals. *Front. Immunol*. 5:459. doi: 10.3389/fimmu.2014.00459). Ref (Buchmann 2014)

Yet they are interesting organisms to study, because they are relatively simple, lack adaptive immune responses, and have immune competent cells known as hemocytes or phagocytic hemocytes representing a certain level of similitude with vertebrate macrophages. Investigating the immune or phagocytic response in such "simple" organisms may shed light on some immune mechanisms that vertebrates have inherited from invertebrates.

6.2 Days of Future Past

In our opinion, one of the most important and intriguing functions of macrophages is phagocytosis, and, quite interestingly, the process of phagocytosis was discovered using an invertebrate animal model. Phagocytosis is the capacity of cells to engulf particles, viruses, and bacteria through the engagement of pattern recognition receptors (PRRs) and actin polymerization and to destroy them in intracellular vacuoles called phagosomes (Gordon 2016; Kinchen and Ravichandran 2008; Sellge and Kufer 2015). The principle of phagocytosis was discovered more than a century ago by Ilya Ilitch Metchnikov, a zoologist and microbiologist, better known by his French name Élie Metchnikoff (awarded the Nobel Prize in Physiology or Medicine in 1908). This major discovery was made using starfish, as Metchnikoff had a keen interest in studying digestion in primitive organisms (Mechnikov 1905). According to legend, he was using a rose thorn to tickle starfish larvae when he observed the recruitment of amoeboid circulating cells, which were engulfing or trying to engulf the rose thorn. Certainly, the work of Metchnikov is not restricted to phagocytosis (Gordon 2008), as he also investigated the inflammatory recruitment process and the digestion of bacteria and probiotics by cells, but these various stories go beyond the scope of this review. What is of interest here is that a mechanism observed in primitive organisms, which had survived for at least 500 million years, may be conserved in complex organisms such as *Homo sapiens*, which evolved only 1.2 million years ago. This highlights the fact that we may be able to better understand immunity and discover new immune functions using invertebrate models. Different functional aspects of phagocytes have been discovered and studied in several vertebrate and invertebrate animal models over the past 100 years. Among them, arthropods and mollusks are the preferred invertebrate models that have revealed several conserved mechanisms and machinery involved in phagocytosis.

6.3 Insects

Insect hemocytes are usually described for their function and role in immunity. The use of model organisms such as Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola has led to the identification of

five types of hemocytes based on morphology: prohemocytes, granulocytes, plasmatocytes, spherulocytes, and oenocytes (de Azambuja et al. 1991; Luckhart et al. 1992; Butt and Shields 1996; Hernandez et al. 1999; da Silva et al. 2000; Lavine and Strand 2002). Furthermore, the characteristic features of different hemocytes have been identified. Granulocytes and plasmatocytes are adherent cells that represent more than 50% of the hemocyte population. It was suggested that oenocytes are probably involved in the melanization process as they contain cytoplasmic phenoloxidase, whereas spherulocytes transport the components required for cuticle fabrication. Plasmatocytes are defined as phagocytic hemocytes in *Drosophila*, whereas in mosquitoes, granulocytes function as phagocytes (Elrod-Erickson et al. 2000; Hillyer and Strand 2014). Because of the difficulty in identifying and classifying the different types of hemocytes, it is important to note that the particular type of hemocytes reported to be phagocytic varies among insect taxa. Phagocytic hemocytes have not only been identified in insects, but they have also been reported in crustaceans such as crayfish (Giulianini et al. 2007) and shrimps (Jauzein et al. 2013) as well as marine bivalve mollusks such as mussels, oysters, and clams (Canesi et al. 2002; Matozzo and Bailo 2015; Wang et al. 2016).

In insects, the recognition of microbes by phagocytic hemocytes requires the presence of PRRs, similarly to vertebrates. Several factors have been identified as PRR candidates in insects. Indeed, the existence of lectins, hemolin, lipopolysaccharide-binding protein, peptidoglycan recognition protein (PGRP) (Dodd and Drickamer 2001; Dziarski and Gupta 2006; Su et al. 1995), and thioester-containing protein (αTEP1) (a complement homolog) (Blandin and Levashina 2004) has been reported. Some of these factors have been shown to contribute to the phagocytic properties of hemocytes. For example, blocking *Bombyx mori* lipopolysaccharide-binding protein with antibodies greatly reduces the capacity of *B. mori* to fight against *Escherichia coli* (Koizumi et al. 1999). Silencing of αTEP1 via RNA interference reduces the phagocytosis of Gram-negative bacteria by hemocytes in *Anopheles gambiae* (Levashina et al. 2001).

The fruit fly *Drosophila melanogaster* is used as a model organism in several scientific fields, including immunology. Compared to other invertebrates, the immune system of *Drosophila* has been explored in detail, because these flies are easy to cultivate, produce numerous progenies, and have a short generation time (10 days at 25 °C). In addition, the genome of the fruit fly has been entirely sequenced and revealed a high degree of similarity with several mammalian genes, suggesting the significant conservation of many biological processes. Moreover, several methods are available to genetically modify *Drosophila* or perform functional genetic screens. Additionally, the *Drosophila* genome has lower complexity and redundancy—features that facilitate the appearance of a phenotype when genetic mutations are induced. As *Drosophila* does not possess an acquired immune system, it relies on innate immunity consisting of cellular and humoral responses involving phagocytic hemocytes (Kounatidis and Ligoxygakis 2012; Imler 2014).

In *Drosophila melanogaster*, PGRP-LE has been identified not only as the sensor and inducer of immune responses against infectious bacteria but also as a factor required for microbiota tolerance. In *Drosophila*, the recognition of bacterial

peptidoglycan by PGRP-LE induces an NF-κB-dependent response to pathogens as well as an immune tolerance to microbiota through the upregulation of pirk (poor Imd response upon knock-in), known as the negative regulator of Imd pathway, and PGRP-LB, which in turn negatively regulates the Imd pathway. In flies, the PGRP-LE knockdown leads to the activation of the immune response against microbiota, which can be rescued by the overexpression of PGRP-LB. PGRP-LE functions as a bacterial sensor that induces both balanced responses to infectious bacteria and tolerance to microbiota (Bosco-Drayon et al. 2012). In vertebrates, integrins are known to bind the components of bacterial membranes and contribute to phagocytosis. In *Drosophila,* similar mechanisms are reported for the integrin αPS3/βv, which is required for the phagocytosis of *Staphylococcus aureus* (Nonaka et al. 2013). In *Ceratitis capitata*, hemocytes showed a decrease in their capacity to phagocyte *E. coli* in the presence of *integrin β-subunit* blocking antibody (Foukas et al. 1998).

Finally, the toll receptor, which was first identified in *Drosophila* with the function of maintaining dorsoventral polarity, was then defined as a key factor in the immune response and is present in multiple copies in several invertebrates and vertebrates (Satake and Sekiguchi 2012; Rauta et al. 2014; Medzhitov et al. 1997; Ramet 2012). In *Drosophila*, genetic studies indicate that toll and Imd signaling pathways activate different antimicrobial genes (Myllymaki et al. 2014; Valanne et al. 2014). Interestingly, it has been observed that *Drosophila*, like vertebrates, has the capacity to develop a trained immunity (Pham et al. 2007). In *Drosophila*, toll-like receptors as well as phagocytes seem to play a role in the establishment of trained immunity. Indeed, *Streptococcus pneumoniae*-primed fruit flies are protected against a second infection by *S. pneumoniae*. This protection involves pathogen (*S. pneumoniae*) recognition and phagocytosis via toll-like receptors. Thus, phagocytes are the critical effectors of the primed response (Pham et al. 2007).

Recently, it was demonstrated that another insect, *Pediculus humanus humanus* (body lice), has an immune capacity that relies directly on its hemocyte functions (Coulaud et al. 2015). Indeed, phagocytic hemocytes have been characterized by their ability to adhere to the support, phagocyte latex beads, and produce reactive oxygen species (ROS). ROS and reactive nitrogen species (RNS) have also been identified as part of cellular immune responses in *Drosophila* (Nappi et al. 2000) and *A. gambiae* (Molina-Cruz et al. 2008). In addition, it has been shown that body louse hemocytes eliminate the *E. coli* strain. Interestingly, hemocytes from body lice can hide various pathogens such as *Rickettsia prowazekii* and *Bartonella Quintana* (Coulaud et al. 2015).

6.4 Crustaceans, Marine Bivalves, and Mollusks

Phagocytic hemocytes from bivalves and mollusks have also been extensively investigated, because the understanding of immunity in these organisms is greatly motivated by economic benefits. Crustaceans and bivalve mollusks live in an

environment containing microorganisms such as protozoans, fungi, bacteria, and viruses that might act as pathogens. Thus, shrimps, oysters, and mussels might have developed their immune system to detect and eliminate these pathogens.

Like many other invertebrates, the antimicrobial defense in crustaceans and bivalves relies solely on the innate immune system. Their innate immune system is activated by the recognition of pathogen-associated molecular patterns (PAMPs). Several PAMP-recognizing receptors and soluble proteins such as lectins and antimicrobial peptides that activate a phagocytic and humoral response to destroy invading pathogens have been identified. Similarly to insects, in crustaceans and bivalves, the recognition of foreign bodies such as microbes seems to be mostly driven by receptors with homology to PGRP and lipopolysaccharide-binding proteins (Vazquez et al. 2009). More interestingly, hemocytes from bivalve mollusks such as giant clams have active phagocytic cells, which, similarly to mammalian phagocytes, are able to recognize foreign bodies via lectins and chemotaxis (Vasta et al. 1984; Cheng 1975). Various methods are available to assess phagocytosis in bivalves (Blaise et al. 2002). Studies using these methods showed that the engagement of PRR leads to a cascade of events that define the immune response of the phagocytic hemocytes, such as the induction of phagocytosis and the production of antimicrobial peptides, ROS, and RNS.

Antimicrobial peptides play a critical role in invertebrates to keep the infection under control. In the majority of insects, antimicrobial peptides are produced in body fat and secreted into the hemolymph (Yi et al. 2014; Tzou et al. 2000). It was demonstrated that in termites and spiders, antimicrobial peptides are produced by hemocytes. It was also suggested that hemocytes from crustaceans such as crabs and shrimps produce antimicrobial peptides, named callinectin and penaeidins, respectively (Khoo et al. 1999; Destoumieux et al. 1997). Similar peptides derived from hemocyanin have been purified from crayfish hemolymph (Lee et al. 2003; Shi et al. 2014).

Hemocytes from bivalves as well as crustaceans are able to produce ROS to kill microbes and degrade pathogenic microorganisms inside a vacuole (phagosome) that contains lysosomal enzymes (Beaven and Paynter 1999; Bachere et al. 2015). It is interesting to note that phagosome formation and biogenesis in these animals follow a similar pathway to that observed in vertebrates (Ye et al. 2012). *Elliptio complanata* hemocytes exhibit ROS and cyclooxygenase activities in response to *Lyngbya wollei*, a pathogenic filamentous cyanobacterium (Gelinas et al. 2014). In *Chlamys farreri*, RNS produced in response to peptidoglycan stimuli contributes to immune defense (Jiang et al. 2013). The signaling pathway induced by pathogen (*E. coli*) recognition remains poorly investigated in bivalves, although there is some similitude with the vertebrate signaling pathway, since, as demonstrated in *Mytilus galloprovincialis* Lam. hemocytes, MAPK- and STAT-like proteins are involved (Canesi et al. 2003; Ciacci et al. 2010; Gaitanaki et al. 2004).

Toll and Imd signaling pathways represent one of the first lines of innate immune defense in invertebrates such as *Drosophila* (Ramet 2012). Homologs of toll-like and Imd pathway components were identified after RNA sequencing of the crustacean *Caligus rogercresseyi*. For example, toll-3, toll-9, and toll-interacting protein,

as well as Traf6 and IKKβ, were identified (Valenzuela-Munoz and Gallardo-Escarate 2014). Similarly, in the bivalve *Paphia undulata*, the spätzle homolog, an activator of the toll pathway, was identified. In vertebrates, toll-like receptors are able to directly recognize bacterial PAMPs and initiate the immune signaling pathway (Akira et al. 2006). In invertebrates such as *Drosophila*, toll-like receptors do not recognize PAMPs, but they are instead activated by the endogenous ligand spätzle (Weber et al. 2003). However, the contribution of the toll-like and Imd pathways to the immune response engaged during pathogen aggression remains unclear. It is important to remember that toll and spätzle are both known to play a crucial role in the development of *Drosophila*, and thus the similar role of these molecules in crustacean and bivalves cannot be excluded.

6.5 Concluding Remarks

With the exception of *Drosophila*, immune mechanisms and phagocytic functions in invertebrates remain poorly investigated, most likely due to the limited genomic information and genetic tools. However, the situation has started to change more recently with the possibility of conducting deep sequencing of hemocytes (Pauletto et al. 2014; Pons et al. 2014) as well as the development of functional genetic screening methods (You et al. 2012; Sagi et al. 2013; Nandety et al. 2015). The immune process in invertebrates is fascinating because of the absence of adaptive immune response. In these organisms, innate immunity drives a large diversity of immune mechanisms to fight microbes. In addition, because of conservation during the evolution of biological processes between organisms, the use of exotic organisms has become beneficial for the understanding of human biology (Buchmann 2014). The studies of invertebrates have made several breakthroughs in immunology research. To mention but a few, PGRPs were first discovered in the hemolymph of silkworms, leading to the discovery of PGRPs in *Drosophila* and the subsequent identification of PGRPs in humans (Royet et al. 2011). The identification of toll-like receptors in *Drosophila* and the subsequent discovery of their role in recognizing microbes have completely changed the perspective toward innate immunity, which used to be considered as a very crude and unsophisticated immune system (O'Neill et al. 2013). Undoubtedly, many more secrets may still be hidden within these creatures, which are worth exploring. Recently, another primitive invertebrate, the freshwater planarian, has been investigated for its immune response, while the MORN2 (also known as MOPT and BLOCK27) gene, essential for immune defense in planarians and humans, was discovered (Abnave et al. 2014). MORN2 enhances the degradation of bacteria via LC3-associated phagocytosis (Abnave et al. 2014). To date, the study of the immune system in non-vertebrates has provided significant insights into antimicrobial responses and shown the potential to provide clues for the development of new antimicrobial strategies to fight against existing and emerging microbes.

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Part II
Immunobiology of Macrophages

Chapter 7
F4/80 as a Major Macrophage Marker: The Case of the Peritoneum and Spleen

Alexandra dos Anjos Cassado

Abstract Tissue macrophages are a heterogeneous cell population residing in all body tissues that contribute to the maintenance of homeostasis and trigger immune activation in response to injurious stimuli. This heterogeneity may be associated with tissue-specific functions; however, the presence of distinct macrophage populations within the same microenvironment indicates that macrophage heterogeneity may also be influenced outside of tissue specialization. The F4/80 molecule was established as a unique marker of murine macrophages when a monoclonal antibody was found to recognize an antigen exclusively expressed by these cells. However, recent research has shown that F4/80 is expressed by other immune cells and is not equivalently expressed across tissue-specific macrophage lineages, including those residing in the same microenvironment, such as the peritoneum and spleen. In this context, two murine macrophage subtypes with distinct F4/80 expression patterns were recently found to coexist in the peritoneum, termed large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). However, the presence of phenotypic and functional heterogeneous macrophage subpopulations in the spleen was already known. Thus, although F4/80 surface expression continues to be the best method to identify tissue macrophages, additional molecules must also be examined to distinguish these cells from other immune cells.

7.1 Introduction

Macrophages reside in several tissues and constitute a highly heterogeneous cell population in terms of ontogeny, morphology, phenotype, gene signature, metabolism, and function (Mosser 2003; Mantovani et al. 2004; Gordon and Taylor 2005; Gorgani et al. 2008; Davies et al. 2013; Ginhoux and Jung 2014; Gordon et al. 2014; Geissmann 2016). The study of macrophage function dates back to 1882 when Élie Metchnikoff (1845–1916), a noted Russian biologist, introduced the concept of

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phagocytosis after observing that foreign particles were ingested by motile cells. Later, Metchnikoff demonstrated that phagocytic cells play key roles in the host resistance to infections and described them as the body's first line of defense. Furthermore, he showed that susceptibility to infection was associated with a pathogen's resistance to phagocytosis and its survival capacity in the cell cytoplasm (Hirsch 1959). For this research, Metchnikoff was awarded the 1908 Nobel Prize in Physiology/Medicine along with Paul Ehrlich, who proposed the theory of humoral immunity (Gordon 2016).

During the 1960s, research by Cohn and Hirsch was key to understanding the biology and function of mononuclear phagocytes, particularly for development of pioneering murine model studies (Cohn and Hirsch 1960a, b; Hirsch and Cohn 1960). Phagocytic capacity was used to classify several cell types into a system first defined as the reticuloendothelial system, which includes endothelial cells, splenic and lymph node reticular cells, Kupffer cells, splenocytes, and monocytes. Later, the term "reticuloendothelial system" was deemed inappropriate, and the phagocytes and their precursors, except endothelial cells, were classified into the mononuclear phagocyte system (MPS) based on morphology, function, and their production kinetics in the bone marrow (van Furth and Cohn 1968; van Furth et al. 1972; van Furth 1980). Accordingly, van Furth detailed the kinetics of MPS cell production under physiological or inflammatory conditions in the late 1960s (van Furth and Cohn 1968; van Furth and Diesselhoff-Den Dulk 1970; Van Furth et al. 1973). In this consensus, the MPS class included monocytic precursors in the bone marrow, peripheral monocytes, resident macrophages, and dendritic cells (DCs) (Hume 2006; van Furth 1980, 1981; Hume et al. 2002). Moreover, the MPS concept assumes that all tissue macrophages differentiate from bone marrow hematopoietic stem cells (HSCs) via a monocyte intermediary (Volkman 1970; van Furth 1970, 1985; van Furth and Diesselhoff-Den Dulk 1970; Van Furth et al. 1973). However, recent studies have demonstrated that some tissue-resident macrophage populations arise from embryogenic precursors originating in the yolk sac and are maintained through self-renewal (Ginhoux et al. 2010; Davies et al. 2011; Schulz et al. 2012; Yona et al. 2013; Hashimoto et al. 2013).

Mononuclear phagocytes are characterized by their endocytic capacity, cytoplasmic enzymes (lysosomal hydrolases and esterases), and the expression of phagocytic receptors such as Fc and complement receptors (Hume 2006). As dynamic cells, the macrophage plasma membrane is highly modified as a consequence of the fusion processes that occur during endocytosis or phagocytosis. Thus, none of the features described by these authors are exclusive enough to define tissue macrophages or to distinguish them from other phagocytic cells (Hume 2006, 2008). Moreover, the phenotypical identity of macrophages was further complicated with the discovery of DCs (Steinman and Cohn 1973), which share several of the same phenotypic and functional characteristics. Recently, Guilliams et al. proposed a new classification of MPS, under steady state and inflammatory conditions, based mainly on their ontogeny (Guilliams et al. 2014).

Despite the technical difficulties in defining and categorizing the macrophage subpopulations (Geissmann et al. 2010; Geissmann and Mass 2016), their

functional significance in immunity is broadly recognized (Unanue 1997), particularly with respect to their roles in phagocytosis, antigen presentation to T lymphocytes (Villacres-Eriksson 1995), apoptotic cell clearance (Aderem and Underhill 1999), inflammatory resolution (Leibovich and Ross 1975), angiogenesis (Polverini et al. 1977), and tissue remodeling and repair (Werb and Gordon 1975).

In this chapter, we will review the discovery of F4/80; its molecular characteristics, function, and regulatory mechanisms; and their use in distinguishing peritoneal and splenic macrophages.

7.2 F4/80 as a Specific Marker of Murine Macrophages

Macrophages were originally identified by their expression of CD11b until CD11b was later found to also be expressed in other cell populations, such as polymorphonuclear neutrophils, NK cells, DCs, and B lymphocytes (Ross 2000; Shortman and Liu 2002). In 1981, Austyn and Gordon reported a hybridoma clone that produced a monoclonal antibody, named F4/80, directed to a macrophage-specific surface antigen (Austyn and Gordon 1981). This hybridoma was obtained by fusing splenocytes from mice immunized with thioglycolate-induced peritoneal mouse macrophages with a murine myeloma cell line (NS1). The F4/80 antibody did not react with other cells of hematopoietic origin, such as fibroblasts, polymorphonuclear cells, and lymphocytes, which share many of the receptors and surface antigens of macrophages (Austyn and Gordon 1981). Moreover, the F4/80 antibody also did not recognize guinea pig peritoneal macrophages, rat and rabbit bone marrow-derived macrophages, or human monocytes and lung macrophages (Austyn and Gordon 1981).

The F4/80 monoclonal antibody binds to macrophage populations in H-2 haplotype mouse strains, including ASN (a), C57BL/6 (b), BALB/c (d), DBA/2 (d), CBH (k), CBA (k), and ASW (s), indicating that it does not detect polymorphic human leukocyte antigen (HLA) determinants (Austyn and Gordon 1981). Subsequently, the utility of F4/80 as a specific marker for tissue macrophages was confirmed in various organs and tissues, including the kidney (Hume and Gordon 1983), bone marrow (Hume et al. 1984b), epithelia (Hume et al. 1984c), lymphoid organs (Hume et al. 1983b), retina (Hume et al. 1983a), and endocrine organs (Hume et al. 1984a). However, it was later verified that Langerhans cells (Hume et al. 1983b) and eosinophils (McGarry and Stewart 1991; Ghosn et al. 2010; Cassado Ados et al. 2011) express F4/80, making it more difficult to distinguish macrophages from other hematopoietic cell populations. including the kidney (Hume and Gordon 1983), bone marrow (Hume et al. 1984b), epithelia (Hume et al. 1984c), lymphoid organs (Hume et al. 1983b), retina (Hume et al. 1983a), and endocrine organs As previously mentioned, CD11b and F4/80 are often used as macrophage markers with the understanding that their expression is not homogeneous among the various macrophage subtypes. Additional surface markers should also be analyzed to identify cell types with little to no F4/80 expression, such as

alveolar macrophages, central nervous system macrophages, splenic marginal zone (MZ) macrophages, and Kupffer cells (Maus et al. 2001; Gordon and Hirsch 1982; Gordon et al. 1988; Movita et al. 2012). The presence of macrophage subpopulations with distinct levels of F4/80 expression in the same microenvironment indicates that the heterogeneity is not exclusively determined by environmental factors, as is the case of the peritoneum and spleen (Taylor et al. 2005a; Cassado Ados et al. 2015). Thus, CD11b and F4/80 expression should be employed in association with other immune cell markers to distinguish between resident macrophage populations.

7.3 Structure and Function of the F4/80 Molecule

F4/80 is a 160-kDa glycoprotein with an epithelial growth factor (EGF)-like extracellular domain and a seven-transmembrane motif (TM7), which are similar to those observed in G protein-coupled receptors (McKnight and Gordon 1996). Based on this modular structure, F4/80 is classified as a typical member of the EGF-TM7 receptor family, which includes the EGF module-containing mucin-like hormone receptors (EMR) 1–4, EGF-TM7-latrophilin-related protein (ETL), and CD97 (McKnight and Gordon 1996, 1998). Figure 7.1 shows a schematic molecular structure of the F4/80 molecule.

The EGF-like domain is present in a wide variety of cell surface proteins involved in adhesion, receptor-ligand interactions, extracellular matrix structure, and cell fate determination (Campbell and Bork 1993). The TM7 domain mediates signal transduction downstream of a wide range of exogenous stimuli, including hormones, cytokines, derivatives of amino acids peptides, neurotransmitters, and sensory stimuli such as photons, flavors, and odors (Pierce et al. 2002; Bockaert and

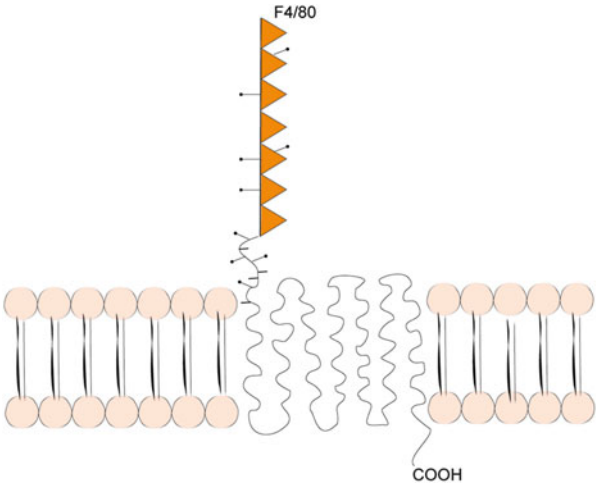

Fig. 7.1 Schematic molecular structure of the F4/80 molecule. F4/80 glycoprotein that consists of an epithelial growth factor (EGF)-like extracellular domain and a seven-transmembrane motif (TM7)

Pin 1999). The existence of specific ligands for many EGF-TM7 receptors (Hamann et al. 1996; Stacey et al. 2001, 2002, 2003) suggests that F4/80 may have a similar function in cell adhesion, which in turn may influence tissue distribution, development, and function in vivo.

Generally, the exclusive expression of a surface molecule in any cell type raises the question of its functional role, as has been the case for F4/80 expression in macrophages. Interestingly, the addition of anti-F4/80 antibodies to splenocyte cultures stimulated with heat-killed *Listeria monocytogenes* resulted in decreased TNF-α, IL-12, and IFN-γ production and increased IL-1 and IL-10 levels, suggesting that F4/80 may mediate an interaction between macrophages and splenic NK cells under inflammatory conditions (Warschkau and Kiderlen 1999).

A germ line of mice expressing Cre recombinase under the direct control of the F4/80 promoter (F4/80-Cre knock-in) generated healthy and fertile animals (Schaller et al. 2002). Homeostatic development of $F4/80^{cre/cre}$ macrophages in this model was normal, and, during infection by *L. monocytogenes*, both the microbicidal response of macrophages and the induction of a T-independent B cell response were not affected by the absence of F4/80, indicating that F4/80 expression in macrophages is dispensable for their development and functions (Warschkau and Kiderlen 1999). Later, another study confirmed that macrophage development occurs independently of F4/80; however, its expression by antigen-presenting cells (APCs) is required to induce regulatory T cell differentiation in a model of anterior chamber-associated immune deviation (ACAID) (Wilbanks and Streilein 1991; Wilbanks et al. 1991; Hara et al. 1992). Additionally, $F4/80^{-/-}$ animals were unable to suppress an antigen-specific delayed-type hypersensitivity response in a mouse model of oral tolerance (Lin et al. 2005). Nevertheless, the adoptive transfer of $F4/80^{+}$ antigen-presenting cells was sufficient to restore peripheral tolerance in both animal models (Schaller et al. 2002).

7.4 Regulation of F4/80 Expression

F4/80 expression is strongly regulated by cell location, interactions with other cells, differentiation stage, and activation profile. For example, blood monocytes express F4/80 at a lower level than their mature counterparts (Gordon et al. 1992). Generally, only macrophages located in the T cell-independent areas of secondary lymph tissue show high F4/80 levels, suggesting that T cells or their products may play an important role in regulating the expression of F4/80 (Gordon et al. 1992).

The regulation of F4/80 expression may also be a consequence of the cell activation status as observed by downregulation of F4/80 expression in macrophages infected with Bacillus Calmette-Guerin (BCG), whereas Mac-1 expression levels remain stable. This phenomenon is accompanied by decreased mannose and Fcγ receptor expression and increased major histocompatibility complex (MHC) expression, implying that these changes are directly related to cellular activation (Ezekowitz et al. 1981). Moreover, the expression of these molecules is tightly

regulated for over 60 h post-inoculation, suggesting that BCG infection generates a stable change in the surface phenotype of peritoneal macrophages (Ezekowitz et al. 1981). A similar mechanism is observed in macrophages stimulated with exogenous IFN-γ, although the response is less pronounced than that following intraperitoneal BCG infection (35 versus 80%) (Ezekowitz and Gordon 1982). Moreover, Langerhans cells show decreased F4/80 expression after antigen uptake and migration to the local draining lymph node (Nussenzweig et al. 1981). These studies alone could explain the presence of macrophage populations with distinct F4/80 levels in the same microenvironment. However, the hypothesis of the coexistence of macrophage populations is further supported by the presence of subpopulations with differential F4/80 expression in naïve animals as observed in the peritoneum and spleen.

7.5 Peritoneal Macrophages

It has long been established that peritoneal macrophages express heterogeneous levels of F4/80, depending on their differentiation and activation states (Taylor et al. 2003). Peritoneal macrophages isolated from naïve animals are often classified as tissue-resident cells and exhibit an F4/80^{high} phenotype, whereas those from thioglycolate-elicited mice show a monocyte-derived F4/80$^{+/int}$ phenotype and are called inflammatory macrophages (Taylor et al. 2003). As such, F4/80 downregulation in resident peritoneal macrophages after stimulation has also been used to justify the presence of distinct cell populations (Ezekowitz et al. 1981; Ezekowitz and Gordon 1982). However, this concept was refuted by the discovery of two macrophage subtypes with differential F4/80 expression in naïve mice (Ghosn et al. 2010). A detailed analysis of these macrophage subtypes by 11-color flow cytometry revealed unique phenotypic, morphological, and functional characteristics (Ghosn et al. 2010). As a result, F4/80^{high} and F4/80^{low} peritoneal macrophages were defined as Large Peritoneal Macrophage (LPM) and Small Peritoneal Macrophage (SPM), respectively, based on morphology. Interestingly, CD11b expression showed a profile similar to that of F4/80 (Ghosn et al. 2010; Cassado Ados et al. 2011).

In fact, the vast majority (~90%) of F4/80^{+} cells in the peritoneum of C57BL/6, BALB/c, FVB/N, RAG$^{-/-}$, 129/S6, and SJL/J mice displayed high F4/80 expression (Ghosn et al. 2010). Sequentially to these findings, our group proposed a simple method to distinguish peritoneal macrophage subtypes by flow cytometry using only four cell surface markers associated with the Side Scatter (SSC) and Forward Scatter (FSC) parameters. Then, after exclusion of doublets cells, CD19^{high} and CD11c^{high} cells, dual F4/80 and MHCII (IA-b) analysis effectively defines three distinct subpopulations, i.e., F4/80^{high}IA-b^{neg}, F4/80^{low}IA-b^{high}, and F4/80^{low}IA-b^{neg}, which correspond to LPMs, SPMs, and granulocytes, respectively (Cassado Ados et al. 2011). These data demonstrate that it is still possible to find non-macrophage cells in the peritoneal F4/80^{+} population; additionally, macrophages and granulocytes are

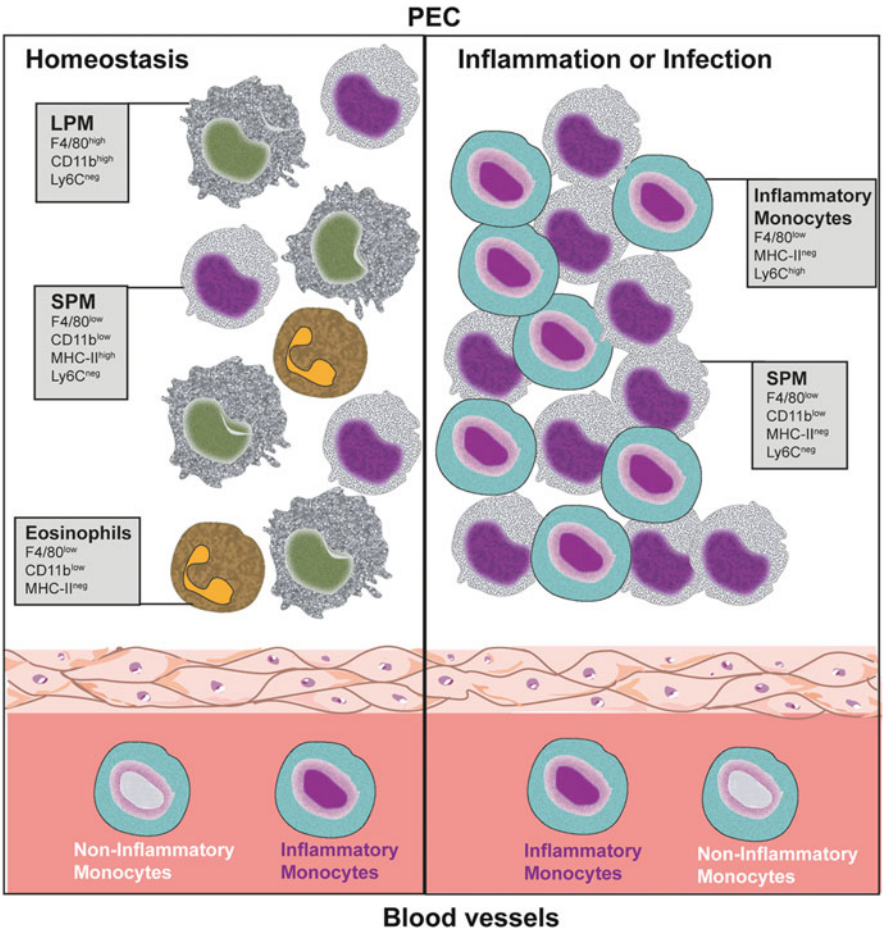

Fig. 7.2 Summary of the peritoneal F4/80^{+} cell phenotype. In healthy adults, most peritoneal cells are F4/80^{high} Large Peritoneal Macrophages (LPMs), whereas the remainder consists of F4/80^{low} Small Peritoneal Macrophages (SPMs) and eosinophils. However, upon inflammatory initiation, expansion of SPMs and monocytes is accompanied by LPM migration into the omentum

easily distinguished by use of appropriated surface markers, in addition to F4/80 and the SSC parameter. Figure 7.2 shows the distribution of F4/80^{+} cells in the peritoneum.

The propensity for macrophages to adhere to tissue culture plastic is often exploited for their specific isolation. When this method is utilized, LPMs generally adopt classical macrophage morphology with abundant cytoplasm and prominent vacuoles, whereas SPMs in culture exhibit polarized morphology with dendrites similar to those found in DCs (Cassado Ados et al. 2011). A complex phenotypic analysis revealed that SPMs display increased expression of IA-b, CD62L, and the Dectin-1 and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) endocytic receptors. Conversely, LPMs show higher

levels of pattern recognition receptors (PRRs) and costimulatory molecules (Cassado Ados et al. 2011; Ghosn et al. 2010). Nevertheless, both subtypes are capable of phagocytosis, even though SPMs appear to be more adept at this function in vivo (Cassado Ados et al. 2011; Ghosn et al. 2010). Despite these differences, various stimuli, such as zymosan, *Trypanosoma cruzi*, thioglycolate, and lipopolysaccharide (LPS), induce similar changes in the peritoneum, resulting in marked LPM disappearance, SPM expansion, and monocyte infiltration (Cassado Ados et al. 2011, 2015; Ghosn et al. 2010; Okabe and Medzhitov 2014). Moreover, stimulation with zymosan or *T. cruzi* induces cell renewal in the peritoneal compartment, marked by decreased β-galactosidase (β-Gal) staining in adherent cells. This effect is accompanied by an increase in NO production and IL-12^{+} cells, mostly within the SPM population (Cassado Ados et al. 2011).

The disappearance of LPMs from the peritoneum has been extensively described as the macrophage disappearance reaction (MDR) during delayed-type hypersensitivity (DTH) and acute inflammatory processes. This reaction is often related to the tissue adhesion capacity of the macrophages, migration into draining lymph nodes, or cell death; however, LPMs migrate from the peritoneum into the omentum after intraperitoneal stimulation (Okabe and Medzhitov 2014), explaining their disappearance after stimulation with LPS, zymosan, and thioglycolate (Davies et al. 2011; Ghosn et al. 2010; Cassado Ados et al. 2011; Cain et al. 2013; Rosas et al. 2014; Okabe and Medzhitov 2014). The unique ability of LPMs to induce gut-associated lymphoid tissue (GALT)-independent IgA production by peritoneal B-1 is regulated by the presence of RA and TGF-B2 in the omentum (Okabe and Medzhitov 2014).

The molecular pathways governing transcriptional regulation in SPMs remain largely uncharacterized; however, studies from Medzhitov's group have characterized the mechanisms involved in the molecular LPM regulation (Okabe and Medzhitov 2014). The GATA6 transcription factor is selectively expressed by LPMs in response to RA presence and regulates peritoneal macrophage-specific gene expression, as well as cell proliferation and survival (Okabe and Medzhitov 2014). Accordingly, a substantially smaller LPM population was observed in the peritoneum of myeloid- and macrophage-specific GATA6 conditional knockout mice (Okabe and Medzhitov 2014). Similarly, mice deficient in the RA precursor vitamin A exhibit a decreased number of GATA-6^{+} LPMs that can be rescued by intraperitoneal stimulation with all-trans retinoic acid. Moreover, GATA-6$^{-/-}$ macrophages present altered proliferation during peritoneal inflammation (Okabe and Medzhitov 2014; Rosas et al. 2014).

The coexistence of several resident cell populations in the peritoneum, including macrophages, B cells, DCs, eosinophils, mast cells, neutrophils, T cells, natural killer (NK) cells, and NKT invariant cells, raises questions regarding the suitability of current peritoneal macrophage isolation and characterization protocols. Early phagocyte biology studies already utilized peritoneal macrophages, as this population can be easily obtained 3–5 days after intraperitoneal injection of thioglycolate (Cassado Ados et al. 2011, 2015; Ghosn et al. 2010; Okabe and Medzhitov 2014). The development of refined experimental techniques has addressed these concerns

and unveiled the diversity of cells residing in the peritoneum. For example, the widely employed adhesion protocol for macrophage isolation has been challenged by some groups. Schleicher and co-workers demonstrated that soluble factors previously thought to be derived from peritoneal macrophages were, in fact, contaminants in T lymphocyte and NK cell culture.

In light of these findings, flow cytometry appeared to be sufficient to identify peritoneal macrophages, but it was also associated with technical limitations with the presence of doublets and the expression of supposedly macrophage-specific markers in other immune cells (Ghosn et al. 2010; Cassado Ados et al. 2011, 2015). Cell doublets can be formed when a macrophage adheres to another macrophage or lymphocyte and are viewed as a single event in single cell sorting. Doublets can be easily excluded during flow cytometry analysis by using size parameters. Thus, peritoneal macrophages cannot be isolated and distinguished exclusively by adherence protocols or F4/80 expression, which may confound experimental results owing to the presence of other cell types. Currently, it is recognized that some of these problems during analysis of peritoneum-resident cell populations can be avoided or minimized using an antibody panel with specific monocytic and lymphoid markers.

7.6 Splenic Macrophages

The spleen is the largest secondary lymphoid organ in the body and ensures the development of immune responses to blood-borne pathogens. The splenic microarchitecture is divided into three parts: the red pulp, the white pulp, and the marginal zone (MZ) that separates them (den Haan et al. 2012; Mebius and Kraal 2005). The localization of macrophages within these areas defines their function in homeostasis maintenance and immune response development. Most splenic macrophages act as sentinels in the pathogen clearance from peripheral blood and can induce pro- or anti-inflammatory immune responses based on surrounding cues (Borges da Silva et al. 2015b; den Haan and Kraal 2012; Kurotaki et al. 2015). In this section, we discuss the distinct macrophage subpopulations within the spleen and their functional significance in host immunity. Figure 7.3 summarizes the microanatomic location of the splenic macrophage subsets and their phenotype.

7.6.1 Red Pulp (RP) Macrophages

Red pulp (RP) macrophages are associated with reticular cells and are in direct contact with the blood circulation, forming a network within the red pulp. These cells are key mediators of erythrocyte clearance and phagocytosis of blood-borne microorganisms and are thus considered blood sentinels (Borges da Silva et al. 2015b; den Haan and Kraal 2012; Kurotaki et al. 2015). RP macrophages are

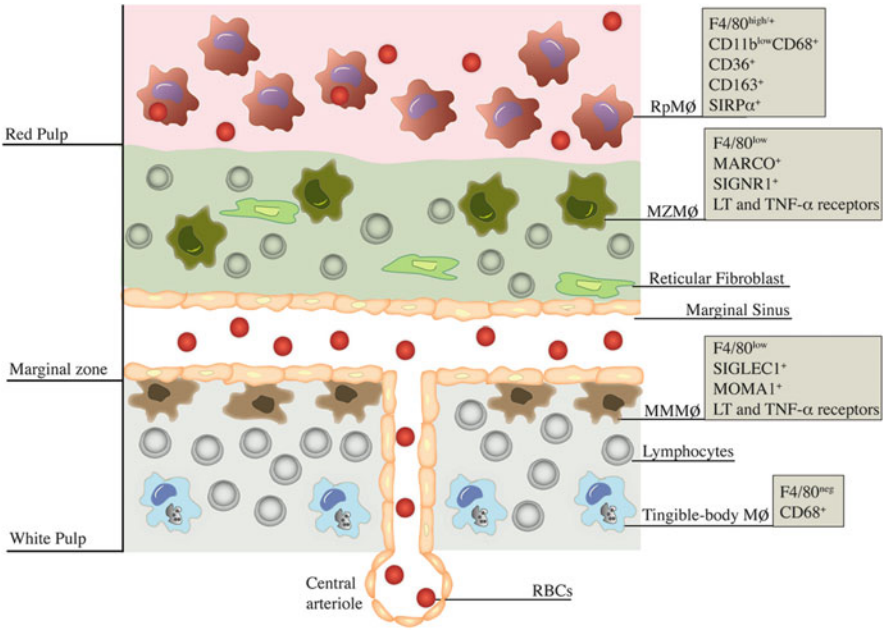

Fig. 7.3 Localization and phenotype of splenic macrophage subtypes. Broad schematic representation of the red pulp (RP), marginal zone (MZ), metallophilic, and white pulp macrophage phenotypes and their localization within the spleen

distinguished from other splenic macrophage populations by their increased F4/80 and CD68 expression and low levels of CD11b (Kohyama et al. 2009; Taylor et al. 2003). They also express high levels of adhesion molecules, including α-integrins and vascular cell adhesion molecule-1 and MHC class I and II (Kurotaki et al. 2011; Taylor et al. 2005b; Kohyama et al. 2009), but are devoid of C–C chemokine receptor 2 (CCR2) and CD80/CD86 costimulatory molecule expression (Kurotaki et al. 2011).

RP macrophages perform several subtype-specific functions that require cell surface receptor expression, such as erythrocyte clearance (Kohyama et al. 2009), blood-borne pathogen phagocytosis (De Jesus et al. 2008; Kirby et al. 2009; Salcedo et al. 2001; Borges da Silva et al. 2015a), peripheral tolerance (Kurotaki et al. 2011), and iron homeostasis (Mebius and Kraal 2005; Kovtunovych et al. 2010; Vidal et al. 1995; Fritsche et al. 2003; Flo et al. 2004). For instance, the removal of apoptotic erythrocytes occurs as a result of a conformational change of CD47 expressed by RP macrophages that activates the phagocytic process (Fritsche et al. 2003). After erythrophagocytosis, iron from the degraded red blood cells is recycled by RP macrophages (Lanoue et al. 2004), and the hemoglobin released into the bloodstream after intravascular disruption of erythrocytes is eliminated through CD163 receptor-mediated endocytosis (Kristiansen et al. 2001). Additionally, RP macrophages secrete cytokines, including IL-10 and TGFβ, to induce

regulatory T cell activation and limit unnecessary immune activation (Kurotaki et al. 2011). RP macrophages are also important mediators of pathogen recognition, particularly for bacterial and fungal infections resulting from *Streptococcus pneumoniae* (Kirby et al. 2009), *Salmonella typhimurium* (Salcedo et al. 2001), and *Cryptococcus neoformans* (De Jesus et al. 2008) or parasitic *Plasmodium*. The recognition of these pathogens occurs via pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), NOD-like receptors (NLRs) (Mebius and Kraal 2005), scavenger receptor CD36 (Flo et al. 2004; Schnitzer et al. 1972; Gautier et al. 2012; Kurotaki et al. 2011), and CD163 (Mebius and Kraal 2005; Seiler et al. 1997; Vanderheijden et al. 2003).

RP macrophages coexist in the red pulp with other immune cells, including DCs, monocytes, granulocytes, NK cells, and lymphocytes. Splenic monocytes are F4/80 ^{low}CD11b^{high} (Swirski et al. 2009), which distinguishes them from F4/80^{+} RP macrophages. However, some RP macrophages display the CD11c^{+} phenotype (Borges da Silva et al. 2015a), which is also shared with splenic DCs.

7.6.2 Marginal Zone (MZ) Macrophages

Two macrophage populations are found in the splenic MZ: metallophilic macrophages and MZ macrophages (Elomaa et al. 1995; Geijtenbeek et al. 2002; Taylor et al. 2005a; Kurotaki et al. 2015). Both subtypes are F4/80$^{low/neg}$, express lymphotoxin and TNF-α receptors (Engwerda et al. 2002; Mackay et al. 1997), and are phenotypically identified by distinct markers. Specifically, MZ macrophages are characterized by high levels of C-type lectin, SIGN-related 1 (SIGNR1), and the class A scavenger receptor macrophage receptor with collagenous structure (MARCO) (Elomaa et al. 1995; Geijtenbeek et al. 2002), whereas metallophilic macrophages express sialic acid marker-binding Ig-like lectin-1 (Siglec-1) and MOMA-1 (Kraal and Janse 1986).

Because of their specific location and receptor expression, both macrophage populations are capable of recognizing and responding to a variety of circulating pathogens and apoptotic cells (Ram et al. 2010; Schmidt et al. 1993). For instance, the SIGN-R1 and MARCO receptors on MZ macrophages recognize encapsulated bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Elomaa et al. 1995; Geijtenbeek et al. 2002; Kang et al. 2004; Lanoue et al. 2004; Koppel et al. 2005), whereas CD169 on metallophilic macrophages has been implicated in the uptake of *Neisseria meningitidis* (Chen et al. 2010; Pluddemann et al. 2009; Jones et al. 2003), *Campylobacter jejuni* (Heikema et al. 2010), and *T. cruzi* (Monteiro et al. 2005). Similarly, both MZ macrophage subsets are directly involved in the removal of apoptotic cells (Miyake et al. 2007) via the scavenger A receptor (Wermeling et al. 2007) and the Treml-4 and Tim-4 receptors (Hemmi et al. 2009; Wong et al. 2010). All of these receptors display an inhibitor signaling mechanism, which is essential for peripheral immune tolerance and whose disruption can lead to development of autoimmune disease in mice and humans (McGaha et al. 2011).

Furthermore, both types of MZ macrophages facilitate the adaptive immune response through various mechanisms (den Haan and Kraal 2012). Metallophilic macrophages assist in the T cell-dependent responses, whereas MZ B lymphocytes require stimulation from macrophage SIGN-R1 to effectively induce IgM-mediated responses (Koppel et al. 2008). A similar phenomenon occurs with follicular B cells and their products, which promote the localization of metallophilic macrophages within the splenic MZ through secretion of lymphotoxins α and β (Nolte et al. 2004).

7.6.3 White Pulp (WP) Macrophages

White pulp (WP) macrophages localize to the splenic white pulp and express CD68, but are devoid of F4/80 expression and other receptors typically expressed by their MZ counterparts (Kurotaki et al. 2015). Although little is known about WP macrophages, their presence in T and B cell regions suggests a likely involvement in antigen presentation and/or the clearance of apoptotic lymphocytes that arise from the germinal center after somatic hypermutation and isotype switching (Kurotaki et al. 2015). These macrophages contain numerous phagocytosed lymphocytes, resulting in the accumulation of condensed nuclear material known as "tingible bodies" and are thus termed tingible body macrophages (Kurotaki et al. 2015).

7.7 Conclusions

Macrophages are specialized cells that reside in different tissues, forming individual subpopulations marked by differences in origin and gene signature, as well as distinct phenotypic, functional, and metabolic characteristics. Historically, CD11b expression was used to identify tissue macrophages, until the discovery of F4/80 as a unique macrophage marker. Although the vast majority of tissue macrophages express F4/80, some populations exhibit low or undetectable expression of this molecule. This difference can even be observed in macrophages that coexist in the same microenvironment, such as in the peritoneum and spleen. The sole use of these markers to designate tissue macrophages is inappropriate, as other myeloid cells are also known to express CD11b and F4/80. Peritoneal macrophages continue to serve as an important experimental tool to study macrophage function and biology, despite the recent identification of two diverse cell populations in the peritoneal cavity. In contrast, the presence of distinct splenic macrophage populations has long been recognized, and, although they are not commonly used as an experimental tool, these populations are crucial for homeostasis maintenance, development of immunity against circulating pathogens, and peripheral tolerance. Thus, tissue macrophage populations must be clearly identified before experimental use to avoid unintended data artifacts. The use of F4/80 and CD11b associated with

other myeloid and lymphoid markers can definitively distinguish macrophages from other peritoneal and splenic cells.

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Chapter 8
Immunobiology of Nitric Oxide and Regulation of Inducible Nitric Oxide Synthase

Martin Lee, Kevin Rey, Katrina Besler, Christine Wang, and Jonathan Choy

Abstract Nitric oxide (NO) is a bioactive gas that has multiple roles in innate and adaptive immune responses. In macrophages, nitric oxide is produced by inducible nitric oxide synthase upon microbial and cytokine stimulation. It is needed for host defense against pathogens and for immune regulation. This review will summarize the role of NO and iNOS in inflammatory and immune responses and will discuss the regulatory mechanisms that control inducible nitric oxide synthase expression and activity.

8.1 Nitric Oxide

Nitric oxide (NO) is a small bioactive gas with a plethora of functions in cell biology. NO was first identified to mediate arterial vasodilatation (Furchgott and Zawadzki 1980; Ignarro et al. 1987; Palmer et al. 1987). In the immune system, NO expression was initially observed in macrophages after cytokine stimulation and microbial infection, suggesting a role for NO in host defense against pathogens (Marletta et al. 1988; Nathan 1992). A decade after the implication of NO in the control of biological processes, three different nitric oxide synthase (NOS) enzymes with distinct tissue expression and function were identified and cloned: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (Bredt and Snyder 1990; Lamas et al. 1992; Lowenstein et al. 1992; Lyons et al. 1992; Xie et al. 1992; Geller et al. 1993; Chartrain et al. 1994). All NOSs synthesize NO through the conversion of L-arginine, NADPH, and O_2 to L-citrulline, $NADP^+$, and NO (Knowles and Moncada 1994). We now know that NO is synthesized by NOSs in multiple cell types and has an incredible range of cellular effects due to extensive regulation of its production and signaling properties.

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8.1.1 Nitric Oxide Signaling

The first mechanism by which nitric oxide was discovered to regulate protein function was through the binding of NO to heme prosthetic groups and metal cofactors in metalloproteins to form a ferrous-nitrosyl complex. Prominent examples of proteins subject to this form of direct NO signaling include soluble guanylyl cyclase (sGC), hemoglobin, cytochrome P450, and nitric oxide synthases (Wink and Mitchell 1998). NO binding to sGC activates this enzyme, leading to the downstream generation of the intracellular secondary messenger cGMP. In contrast to the activating effect of NO on sGC function, NO binding to heme in cytochrome P450 inhibits activity of this enzyme (Wink et al. 1993; Khatsenko et al. 1993; Stadler et al. 1994). NO can also bind and inhibit nonheme metalloproteins such as ribonucleotide reductase, NADH:ubiquinone oxidoreductase, NADH:succinate oxidoreductase, cis-aconitase, and all Fe-S enzymes. This inhibits DNA synthesis and cellular respiration (Nathan 1992; Jorens et al. 1995).

NO reacts with superoxide anion (O_2^-) to form the oxidizing agent peroxynitrite ($ONOO^-$) (MacMicking et al. 1997a). This reaction is physiologically important because phagocytotic cells (i.e., macrophages, polymorphonuclear neutrophils) produce O_2^- as a mechanism to clear pathogens (DeLeo et al. 1999; Lambeth 2004). As such, in macrophages, which produce both O_2^- and NO, these molecules can react to form peroxynitrite (Wink and Mitchell 1998). Although peroxynitrite alone is not highly reactive, its derivative peroxynitrous acid (ONOOH) is highly cytotoxic (Forman and Torres 2001). In the cell, peroxynitrite levels are controlled by glutathione peroxidases, which catalyze the reduction of peroxynitrite to nitrite (Sies et al. 1997; Sies and Arteel 2000). However, NO and peroxynitrite can inhibit the activity of glutathione peroxidase by reacting with its catalytic selenocysteine residue (Asahi et al. 1995; Padmaja et al. 1998). Peroxynitrite can also participate in cell signaling as a secondary messenger through a process called protein nitration, where peroxynitrite reacts with tyrosine residues in proteins to generate 3-nitrotyrosine. Protein nitration may regulate signaling processes by hindering tyrosine phosphorylation (Kong et al. 1996). Nitrotyrosine formation inactivates enzymes such as glutathione reductase, tyrosine hydroxylase, and manganese superoxide dismutase and activates others such as cyclooxygenase (Francescutti et al. 1996; Ara et al. 1998; Yamakura et al. 1998; Landino et al. 1996). Protein nitration is a reversible process and is regulated by putative denitrase enzymes (Kamisaki et al. 1998; Kuo et al. 1999; Osoata et al. 2012). Altogether, NO-dependent generation of peroxynitrite regulates cell toxicity and cellular signaling by distinct mechanisms, likely depending on the cellular bioavailability of this radical.

NO and peroxynitrite can also react with protein thiol groups to form *S*-nitrothiols (SNOs), a process termed *S*-nitrosylation (Forman and Torres 2001; Hess et al. 2005). *S*-nitrosylation of proteins can regulate protein activity, turnover, and folding (Lander et al. 1995; Li et al. 2007; Mitchell et al. 2005). Proteins can be *S*-nitrosylated by exposure to free NO or through a regulated process involving the

transfer of a NO moiety from donor to acceptor molecules. Glutathione and thioredoxin are key regulators of protein *S*-nitrosylation and act as a source of NO to target other proteins for transnitrosylation. *S*-nitrosoglutathione (GSNO) is the main nonprotein *S*-nitrothiol in the cell and functions in equilibrium with other *S*-nitrosylated proteins (Hess et al. 2005). In contrast, thioredoxins provide specificity for target proteins and have dual roles in regulating both the *S*-nitrosylation and *S*-denitrosylation of proteins (Mitchell and Marletta 2005; Benhar et al. 2008).

8.2 Immunobiology of Nitric Oxide

NO can be produced by many cells of the immune system. Macrophages, neutrophils, some types of dendritic cells, and T cells can all be induced to express iNOS in response to various stimuli. Within this context, NO acts as an effector of inflammation for the clearance of bacterial infections and also as a regulator of adaptive immune responses.

8.2.1 iNOS and NO in Pathogen Clearance

iNOS is expressed by macrophages in response to bacterial infections (Bogdan 2001). Studies in humans have identified increased expression of iNOS in patients infected with tuberculosis as compared to healthy controls (Landes et al. 2015). Experimental studies using iNOS-deficient mice determined that they were more susceptible than wild-type counterparts to a wide variety of infectious diseases and sepsis, indicating that production of NO by iNOS is needed for protective immune responses (Alam et al. 2002; Cobb et al. 1999; Poljakovic and Persson 2003; MacMicking et al. 1997b; Rajaram and Nelson 2015; Serbina et al. 2003; Bogdan 2001). The importance of this host defense mechanism is further supported by the identification of microbial components that have evolved to inhibit iNOS activity by sequestering cofactors (Lamont et al. 2013).

The antimicrobial activity of NO is mediated primarily by its toxicity to cells and microbes. Exposure of cells to high levels of NO causes DNA damage in the form of strand breaks and deamination of guanine and adenine (Wink et al. 1991; Nguyen et al. 1992). In many circumstances, some of the cytotoxic effects of NO have been attributed to effects of peroxynitrite, which can cause oxidation and nitrosylation of individual nucleotides (Szabo 1996; Korkmaz et al. 2009; Niles et al. 2006; Wink and Mitchell 1998). Growth of bacterial species is inhibited directly by NO, and this gas increases the bactericidal activity of H_2O_2 that is produced by activated neutrophils and macrophages (Yadav et al. 2014).

8.2.2 *Effects of Nitric Oxide on Cell Viability*

NO is also an important regulator of host cell viability. It can either inhibit or induce cell death. Factors that determine these opposing effects of NO are cell type, its concentration in affected cells, and the redox state of the cell. It is generally believed that low concentrations of NO inhibit cell death and high levels are cytotoxic (Yoshioka et al. 2003; Kim et al. 2005). Apoptosis is a regulated form of cell death that involves the activation of a cascade of cysteine proteases, called caspases, which proteolyze cellular components and induce fragmentation of DNA (Elmore 2007). NO can *S*-nitrosylate the catalytic cysteines within several caspases and in this way inhibit their enzymatic activity (Hess et al. 2005; Jiang et al. 2009; Li and Wan 2013; Dimmeler et al. 1997a, b; Kim et al. 1997). Also, apoptosis can be induced by permeabilization of the mitochondria, a process controlled by proteins of the Bcl-2 family (Zamora et al. 2001; Elmore 2007). NO can prevent mitochondrial permeability through activation of cGMP in the rat heart and in isolated cardiac mitochondria (Borutaite et al. 2009). This could occur through NO-mediated inhibition of expression of the proapoptotic Bcl-2 proteins Bak and BNIP3 and increased expression of Bcl-2 (Zamora et al. 2001; Ciani et al. 2002; Genaro et al. 1995). Finally, low concentrations of NO protect against oxidative damage by inducing expression of heme oxygenase-1 (Wink et al. 1994; Choi et al. 2003; Kim et al. 1995; Liu et al. 2007; Fujii et al. 2010). Inhibition of heme oxygenase-1 enhances the production of reactive oxygen species and increases apoptosis, while overexpression of heme oxygenase prevents these toxic effects (Castilho et al. 2012).

In contrast to the survival effects described above, NO can be cytotoxic to host cells in certain situations. As mentioned, high levels of NO can lead to peroxynitrite formation and to DNA damage. Peroxynitrite formation from high levels of mitochondrial NO causes cytochrome c nitration and release, resulting in apoptosis (Borutaite et al. 1999; Ghafourifar et al. 1999; Cassina et al. 2000). This gas can also induce cell death through the activation of p38 MAPK and JNK signaling pathways (Cheng et al. 2001; Guner et al. 2009; Jin et al. 2014; Kuzushima et al. 2006; Natal et al. 2008; Baek et al. 2015; Hebestreit et al. 1998; Ilmarinen-Salo et al. 2012). Posttranslational modification of some proteins by *S*-nitrosylation can also be cytotoxic. *S*-nitrosylation of GAPDH by iNOS augments binding of GAPDH to Siah1 (an E3 ubiquitin ligase). This complex translocates to the nucleus where Siah1 enhances the ubiquitination and subsequent degradation of nuclear proteins, leading to apoptosis (Hara et al. 2005). Nuclear GAPDH also binds the p300/CREB-binding protein (CBP) and activates p300/CBP through autoacetylation. The GAPDH-p300/CBP complex then stimulates the activation of p53, which induces expression of proapoptotic genes such as PUMA and Bax, resulting in cell death (Sen et al. 2008).

The opposing effects of NO on cell death likely depend on whether they are mediated by direct effects on proteins or through indirect effects caused by the formation of reactive nitrogen species. In an oxygen-rich environment, NO reacts

with O_2 or $O_2\cdot$ to form nitrogen dioxide, dinitrogen trioxide, and peroxynitrite (Ford et al. 1993; Huie and Padmaja 1993). The half-life of NO is dependent on its local concentration and on the rate of these autoxidation reactions. At low concentrations NO is long-lived (100–500 s), allowing this gas to diffuse to interact with target proteins. In contrast, formation of reactive nitrogen species predominates with high concentrations of NO (e.g., from activated macrophages), which can contribute to toxic effects (Ford et al. 1993; Wink et al. 1991; Nguyen et al. 1992). Therefore, low doses of NO have a direct effect on cell survival and proliferation, while high NO concentrations confer indirect effects mediated by the formation of reactive nitrogen species (Wink and Mitchell 1998).

8.2.3 Regulation of Immune Responses by NO

T cells differentiate into separate effector subtypes depending on the context of their activation (Coquet et al. 2015). For CD4 T cells, these subtypes are broadly divided into Th1 cells, which are protective against intracellular pathogens; Th2 cells, which are protective against helminths; and Th17 cells, which are protective against extracellular bacteria and fungi. Regulatory T cells (Tregs) oppose effector T-cell activation and prevent the development of pathological immune responses that can cause autoimmunity (Kim et al. 2009a). iNOS affects the development of Th1 and Th17 cells. Mice deficient in iNOS develop an exacerbated Th1 response after bacterial infection, indicating that NO produced through this enzyme downregulates Th1 cells (Wei et al. 1995. NO may inhibit Th1 responses by attenuating the production of IL-12 in macrophages and through the cytotoxic effect of iNOS-derived NO on memory T cells (Huang et al. 1998; Vig et al. 2004). iNOS expressed by T cells can also inhibit Th17 differentiation in mice by nitrating the transcription factor for Th17 differentiation, RORγt, and reducing the expression of the aryl hydrocarbon receptor (AHR) (Jianjun et al. 2013; Niedbala et al. 2011). In contrast, low concentrations of NO can increase the development of Th1 cells through increasing expression of IL-12R in human T cells and may also induce and maintain human Th17 cells in certain instances (Niedbala et al. 1999; Obermajer et al. 2013). iNOS expression in human T cells also acts in a paracrine manner to amplify the expansion of effector T cells through inhibition of T-cell death (Choy et al. 2007; Choy and Pober 2009). NO also suppresses Treg development induced by TGF-β (Lee et al. 2011). Differences in the immunological effects of iNOS in T-cell biology may be related to distinct effects in mouse versus human cells.

In addition to impacting effector T-cell responses, iNOS modulates antibody responses. iNOS expression is induced in dendritic cells through TLR stimulation, and this is important for the production of IgA antibodies in mucosa-associated lymphoid tissues (Tezuka et al. 2007). However, iNOS expression in lymph node stromal cells and bone marrow inhibits IgG and IgM antibody production by reducing the expression of B-cell-activating factor (BAFF) (Giordano et al. 2014).

Lastly, iNOS regulates the infiltration of immune cells into sites of inflammation by altering cell adhesion. NO reduces the expression of cell adhesion molecules on endothelial cells, thereby inhibiting the transendothelial migration of immune cells into sites of inflammation (Dal Secco et al. 2006; Xu et al. 2013). Nitric oxide can also affect tissue permeability by degrading E-cadherin in cooperation with matrix metalloproteases (MMPs) (Mei et al. 2002).

8.2.3.1 iNOS in Inflammatory Diseases

Given the diverse roles of iNOS and NO in inflammation and immune responses, dysregulation of iNOS is involved in the pathogenesis of several inflammatory and immune-mediated diseases. An exhaustive discussion of iNOS in these conditions is not possible in this chapter, but we highlight below some prevalent diseases that are affected by alterations in iNOS expression and production of NO.

Rheumatological diseases are driven by dysregulation of immune responses that result in inflammatory tissue damage, often to self-antigens. iNOS is upregulated in inflamed tissue sites from patients with systemic lupus erythematosus, rheumatoid arthritis, and psoriasis (Bruch-Gerharz et al. 1996; Dey et al. 2016; Bao et al. 2012; Ding et al. 2014; Oates and Gilkeson 2006). In these conditions, high levels of NO can be tissue damaging (Nowling and Gilkeson 2011; Simonetti et al. 2009). In addition, NO increases the number of mitochondria in lymphocytes from systemic lupus erythematosus patients, and this may lead to dysregulated $Ca2^{+}$ storage and T-cell function (Nagy et al. 2004). Peroxynitrite may also contribute to ssDNA autoantibody generation in systemic lupus erythematosus by modifying thymidine monophosphate, rendering it more immunogenic (Rasheed et al. 2012).

The induction of iNOS by LPS and proinflammatory cytokines in the gut microenvironment contributes to inflammatory bowel diseases (Sasaki et al. 2003; Tun et al. 2014; Kolios et al. 2004). In combination with IL-6, NO reduces epithelial integrity during chronic inflammation (Du Plessis et al. 2013). In a mouse model of colitis, iNOS directly injures the intestine by killing colonic neurons (Venkataramana et al. 2015).

iNOS expression is induced in epithelial cells and macrophages in the lung during inflammation where it contributes to tissue damage, dysfunction, and remodeling. Peroxynitrite formation by iNOS contributes to asthma (Meurs et al. 2003). High iNOS expression is also found in pulmonary fibrosis, emphysema, as well as other airway diseases (Zhao et al. 2012). Progressive damage in the lung can lead to development of chronic obstructive pulmonary disease (COPD), which is characterized by narrowing of airways and destruction of alveoli. COPD patients express high levels of iNOS, TNFα, and nitrotyrosine in the lung (Agusti et al. 2004; de Oca et al. 2005). The development of COPD correlates with changes in iNOS expression in humans, and its inhibition or elimination prevents COPD development in a mouse model (Seimetz et al. 2011). The relationship with NO and COPD is complex, however, as COPD patients have lower levels of NO in their serum than healthy controls (Aydin et al. 2015).

Atherosclerosis is an inflammatory vascular disease that is characterized by the focal development of cholesterol-rich plaques that expand the intima of affected arteries. These plaques contain several immune cells, including macrophages and T cells (Libby et al. 2002, 2013; Pircher et al. 2016). iNOS is expressed in atherosclerotic plaques where it may contribute to the pathological retention and dysfunction of macrophages (Hunter et al. 1999; Huang et al. 2014; Sigala et al. 2010). However, NO can also reduce the progression of atherosclerosis by attenuating macrophage proliferation and reducing mononuclear cell accumulation in plaques through downregulating expression of the cell adhesion molecule VCAM-1 (Brunner et al. 2015; Takata et al. 2013).

8.3 Inducible Nitric Oxide Synthase

NO is synthesized by nNOS, iNOS, and eNOS (Knowles and Moncada 1994). The classifications, functions, and regulation of the three different NOS isotypes are summarized in Table 8.1. Due to the constitutive nature of their expression, nNOS and eNOS are sometimes referred to as constitutive NOSs (cNOSs). Their activities are also dependent on increases in cytosolic Ca^{2+}, which allows for rapid and precise control of NO production in regulating vascular homeostasis by eNOS and neurotransmission by nNOS (Lamas et al. 1992; Bredt and Snyder 1990). In comparison, iNOS expression is normally absent in cells and is induced in response to inflammatory stimuli such as cytokines. Its enzymatic activity is Ca^{2+} independent. Once expressed, iNOS can generate high levels of NO with lasting effects on inflammation and host immunity (Nathan and Xie 1994). These characteristics are not absolute, as all three NOS isoforms have been reported to be regulated by gene expression and to participate in immunity (Bogdan 2001).

All NOS isoforms function as homodimers and synthesize NO through a two-step conversion of L-arginine, NADPH, and O_2 to L-citrulline, $NADP^+$, and NO. In addition, all NOSs require the cofactors heme, tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin for enzyme activity. First, L-arginine is hydroxylated to N^{ω}-hydroxy-L-arginine

Table 8.1 Characteristics of NOS isoforms

	nNOS	iNOS	eNOS
Gene	NOS1	NOS2	NOS3
Basal expression	Yes	Low/no	Yes
Ca^{2+}-dependency	Yes	No	Yes
Main functions	Neurotransmitter, cell signaling	Inflammation, immune modulation	Vasodilation

intermediate. N^{ω}-hydroxy-L-arginine is then oxidized into L-citrulline and NO (Marletta 1993; Stuehr 1999).

The iNOS polypeptide is an oxidoreductase with an N-terminal oxidase domain, a calmodulin-binding domain, and a C-terminal reductase domain (Stuehr 1999; Crane et al. 1998). The N-terminal oxidase domain, a region of approximately 500 residues, binds the cofactors heme and BH_4 and has binding pockets for the substrates L-arginine and O_2. A calmodulin-binding domain lies downstream of the oxygenase core. In eNOS and nNOS, calmodulin binding is reversible, dependent on Ca^{2+}, and triggers electron transfer from the flavin cofactors in the reductase domains to the heme cofactor in the oxygenase domain. For iNOS, calmodulin binding is irreversible and independent of Ca^{2+} flux, allowing iNOS to begin catalysis once assembled (Stuehr 1999). The C-terminal reductase domain spans a region of about 570–625 residues and binds the cofactors FMN, FAD, and the substrate NADPH. During NO synthesis, NADPH donates electrons to FAD, FMN, and finally to the heme-oxygen (Xie et al. 1994a; Stuehr 1999; Marletta 1993).

iNOS expression is induced by many stimuli and is extensively regulated at transcriptional, translational, and posttranslational levels. Importantly, iNOS is regulated differently between species and cell types. While the protein structure of iNOS and the chemistry of NO synthesis are similar between mice, rats and humans, the regulation of iNOS expression is different between laboratory mice/rats and humans (Weinberg et al. 1995). Cultured murine macrophages express high levels of iNOS in response to many stimuli, including LPS and cytokines (Xie et al. 1993, 1994b; Kamijo et al. 1994). Cultured human macrophages do not express iNOS in response to the same stimuli, although iNOS is detected in macrophages from individuals infected with tuberculosis (Padgett and Pruett 1992; Schneemann et al. 1993; Schneemann and Schoedon 2002; Nicholson et al. 1996; Choi et al. 2002). In addition, murine endothelial cells can express iNOS after LPS and cytokine treatment, but human endothelial cells do not because of extensive methylation of the promoter (Chan et al. 2005).

The mouse and human iNOS gene promoters are very different. The transcription factor-binding elements that drive expression in the human iNOS promoter lie between 3 and 13 kb upstream of the transcription start site, while most of the transcription factor elements in the mouse promoter are within 1 kb of the transcription start site (Xie et al. 1994b; Taylor et al. 1998; Marks-Konczalik et al. 1998). Integration of a human iNOS promoter-EGFP reporter system into the genome of mice shows different tissue expression of the human iNOS promoter compared to endogenous mouse iNOS (Yu et al. 2005). In addition, comparisons between the mouse and human iNOS promoter in cultured cells show that the human iNOS promoter, particularly in primary cells, is consistently more heavily methylated (Chan et al. 2005). Below we summarize the cell signaling pathways and regulatory events that control the induction of human iNOS expression (Fig. 8.1).

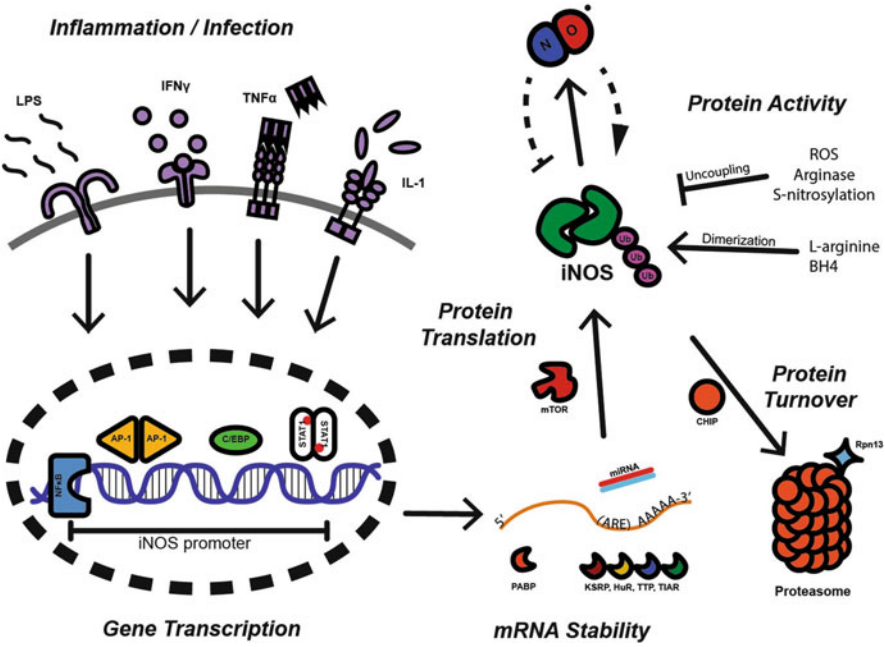

Fig. 8.1 Transcriptional, translational, and posttranslational mechanisms that control iNOS expression and function. iNOS transcription is induced synergistically through a combination of cytokines and/or LPS which leads to the activation of multiple transcription factors. iNOS expression is also regulated posttranscriptionally by cellular factors that affect iNOS mRNA and protein stability. Cellular factors also affect iNOS function by regulating substrate/cofactor availability and enzyme dimerization

8.3.1 Transcriptional Regulation of iNOS Expression

Cytokines induce the expression of iNOS through activation of the iNOS gene promoter. In most human cells, the combined action of several cytokines (such as IL-1β, IFNγ, TNFα) is needed to optimally induce iNOS expression although IFNγ alone can induce iNOS in certain cells (Mellott et al. 2001; Kleinert et al. 1998). Cytokine stimulation initiates signaling cascades that activate transcription factors and resultant gene expression. The human iNOS gene promoter is activated by the cooperative action of several transcription factors (Marks-Konczalik et al. 1998; Taylor et al. 1998). IL-1β and TNFα stimulation leads to the activation and nuclear translocation of NFκB, while IFNγ stimulation results in STAT-1 and IRF-1 activation (Diaz-Cazorla et al. 1999; Kwon et al. 2001; Ganster et al. 2001). In addition to these transcription factors, the human iNOS (hiNOS) promoter also has binding sites for AP-1, C/EBP, CREB, GATA, HIF, KLF6, NF-AT, NRF, Oct-1, PARP1, p53, Sp1, TBE, and TCF (Guo et al. 2016).

NFκB is a major regulator of cytokine-induced iNOS expression. The hiNOS promoter contains multiple NFκB-binding elements at −115 bp and −5.2, −5.5,

−5.8, −6.1, and −8.3 kb (Taylor et al. 1998; Kolyada and Madias 2001; Ganster et al. 2001; Marks-Konczalik et al. 1998; Kristof et al. 2001). In addition to NFκB, the transcription factor AP-1 is activated downstream of MAP kinase pathways that are stimulated by cytokines (Kristof et al. 2001; Hua et al. 2002). There are two AP-1-binding elements in the hiNOS promoter at −5.1 and −5.3 kb (Marks-Konczalik et al. 1998; Kristof et al. 2001). Cytokine stimulation increases AP-1 binding at both loci. However, the two AP-1 elements differ in their ability to confer hiNOS gene inducibility, with the upstream −5.3 kb site being crucial for promoter activation while the downstream −5.1 kb site is less important. Interestingly, an IFNγ response element (γIRE) at −5.3 kb overlaps with the upstream AP-1 element, but mutations of the γIRE outside of the overlapping region do not affect cytokine induction of the iNOS promoter, indicating that AP-1 is the primary factor at this locus (Marks-Konczalik et al. 1998). The upstream AP-1 element at −5.3 kb is also critical for the activity of p300, a transcriptional coactivator involved in cytokine induction of hiNOS gene expression. p300 enhances gene expression by relaxing chromatin structure and by recruiting RNA polymerases (Guo et al. 2016; Kovacs et al. 2003; Schwartz et al. 2003). Overexpression of p300 enhances cytokine-induced iNOS levels, and chromatin immunoprecipitation analysis confirmed that p300 forms a complex with AP-1 and the RNA pol II protein Fra-2 at the −5.3 kb AP-1 binding site (Guo et al. 2016). Another critical transcription factor for human iNOS induction is CCAAT-enhancer-binding proteins (C/EBP) (Kolyada and Madias 2001; Guo et al. 2003). IL-1β induction of hiNOS gene expression requires cooperation between NFκB and C/EBP. A C/EBP binding site at −183/−191 bp was shown to be important for the induction of hiNOS by IL-1β, with C/EBPβ identified as the isoform involved. Mutation of either this C/EBP element or the NFκB element at −115 bp partially reduced iNOS induction by IL-1β, while double mutants completed abrogated iNOS promoter activity (Kolyada and Madias 2001). C/EBP likely contributes to iNOS expression through its ability to recruit and phosphorylate p300 (Kovacs et al. 2003; Schwartz et al. 2003; Saha and Pahan 2006). Finally, the IFN-JAK-STAT pathway is also important in the induction of the hiNOS promoter, as pharmacological inhibition of JAK signaling inhibits cytokine-induced iNOS expression. Two overlapping NFκB and STAT-1 elements are present in the hiNOS promoter at −5.2 and −5.8 kb. The −5.8 kb element is a bifunctional NFκB/STAT-1 element that can bind to either transcription factor, while the −5.2 kb motif only binds STAT-1 (Ganster et al. 2001).

8.3.2 Regulation of iNOS mRNA Translation

Under basal conditions, there is a low level of transcriptional activity of the iNOS promoter without measureable iNOS protein expression, suggesting iNOS mRNA levels are unstable and/or translation is blocked (de Vera et al. 1996; Linn et al. 1997). The untranslated regions (UTRs) of mRNA bind effector proteins and confer resistance or sensitivity to mRNA degradation. In particular, the 3′-UTR of iNOS

contains five cis-acting AU-rich elements (AREs) that are involved in mRNA deadenylation, 5′-decapping, and mRNA degradation (Rodriguez-Pascual et al. 2000; Shyu et al. 1991; Gao et al. 2001). ARE-binding proteins (such as KSRP, HuR, TTP, TIAR) modulate the turnover of ARE-containing mRNAs and can control the recruitment and translocation of target transcripts to the mammalian exosome, a large complex of exoribonucleases that degrade mRNA (Chen et al. 2001; Mukherjee et al. 2002). The KH-type splicing regulatory protein (KSRP) is a key regulator in human iNOS expression. Under basal conditions, KSRP binds the iNOS 3′-UTR and facilitates mRNA degradation. Overexpression of KSRP markedly reduces hiNOS expression, while downregulation of KSRP increases iNOS mRNA stability (Linker et al. 2005). In contrast, the embryonic lethal abnormal vision-like protein Hur is a positive regulator of hiNOS mRNA stability. Overexpression of HuR augments cytokine-induced iNOS mRNA and protein levels, while downregulation of HuR by RNA interference decreases iNOS expression without affecting iNOS promoter activity (Rodriguez-Pascual et al. 2000). Hur binds to the most 3′-located AU-rich element of the iNOS 3′-UTR, the same binding site for KSRP (Rodriguez-Pascual et al. 2000; Linker et al. 2005). Tristetraprolin (TTP) is another positive regulator of iNOS expression. Overexpression of TTP in human cells enhances cytokine-induced iNOS expression, and inhibition of TTP reduces iNOS expression. Cytokines that induce iNOS expression upregulate TTP expression through p38 MAPK and JNK pathways (Fechir et al. 2005a; Korhonen et al. 2007). Unlike HuR, TTP does not directly bind to the human iNOS mRNA but instead interacts with KSRP and the exosome (Fechir et al. 2005a; Linker et al. 2005). In summary, cytokine stimulation enhances iNOS mRNA stability through effects on KSRP, HuR, and TTP. In untreated cells KSRP mediates iNOS mRNA turnover through the exosome. Cytokine treatment increases TTP expression and TTP-KSRP binding, which dislodges KSRP from its iNOS 3′-UTR binding site. As KSRP and HuR compete for the same ARE in the iNOS 3′-UTR, cytokine treatment increases HuR binding, further displacing KSRP and stabilizing the iNOS transcript against turnover (Linker et al. 2005; Fechir et al. 2005a; Rodriguez-Pascual et al. 2000). The T-cell intracellular antigen-1-related protein (TIAR) also binds to the iNOS 3′-UTR and enhances cytokine-induced iNOS mRNA levels (Fechir et al. 2005b).

iNOS mRNA is also subject to regulation by microRNAs (miRNAs), which target specific sequences in the 3′-UTR to promote mRNA degradation and/or block translation. Sequence analysis of the human iNOS 3′-UTR identified five binding sites for miRNA-939. Transfection of cells with this miRNA reduced NO production and iNOS proteins levels, with no effect on iNOS mRNA levels, indicating that miRNA-939 inhibits translation of iNOS without affecting mRNA stability. Cytokines induce miRNA-939 expression, and this acts in a negative feedback manner to downregulate iNOS expression (Guo et al. 2012).

iNOS mRNA stability is also regulated at the 5′-UTR through its interactions with poly(A)-binding protein (PABP), a protein normally involved in mRNA turnover and stability. Downregulation of PABP by RNA interference reduces

cytokine-induced iNOS mRNA levels without affecting human iNOS promoter activity (Casper et al. 2013).

8.3.3 Regulation of iNOS Protein Turnover

Protein levels in a cell reflect the balance between de novo synthesis and subsequent degradation. iNOS protein has a half-life of ~1.6 h in most human cells, and its degradation is mediated by the ubiquitin-proteasome pathway (Musial and Eissa 2001; Kolodziejski et al. 2002, 2004). Rpn13, a 19S proteasome cap-associated protein, is needed for iNOS protein degradation as gene knockdown of Rpn13 prevents iNOS protein degradation by the proteasome (Mazumdar et al. 2010; Huang and Ratovitski 2010). Proteasomal degradation of iNOS also requires polyubiquitination by the E3 ubiquitin ligase COOH-terminus of heat shock protein 70-interacting protein (CHIP) (Chen et al. 2009; Sha et al. 2009). In addition to proteasomal degradation, cells sequester misfolded or aggregated proteins in specialized compartments called "aggresomes." CHIP is also involved in aggresome formation and may be needed for the translocation of iNOS to the aggresome compartment. Together, CHIP promotes iNOS posttranslational turnover through its effects on proteasome-mediated degradation as well as protein sequestration in the aggresome (Sha et al. 2009).

8.3.4 Regulation of iNOS Activity

iNOS is also regulated at the posttranslational level through the control of enzyme catalysis, protein dimerization, and cofactor and substrate availability. Oxidative stress and iNOS *S*-nitrosylation enhance dimer disruption (Milstien and Katusic 1999; Mitchell et al. 2005). Direct binding of NO to the heme cofactor in iNOS inhibits catalysis by blocking the binding of molecular oxygen and interfering with substrate oxidation (Wink and Mitchell 1998; Griscavage et al. 1994). Insufficient BH_4 and L-arginine availability results in NOS uncoupling, which is characterized by reduced NO output and increased ROS/RNS production (Giovanelli et al. 1991; Xie et al. 1996; Baek et al. 1993; Klatt et al. 1995; Heusch et al. 2010; Milstien and Katusic 1999).

iNOS is enzymatically active as a homodimer, and regulation of dimerization is a key step in controlling iNOS activity. iNOS dimerization is a two-step process and requires the binding of FAD, FMN, and BH_4 (Stuehr 1999; Kwon et al. 2001). A functional reductase domain is first assembled with one molecule of FAD and FMN binding each to a monomer of iNOS. This is followed by heme incorporation, dimerization of the two oxygenase domains, and BH_4 binding (Stuehr 1999). BH_4 binding is crucial for NOS activity because it allosterically controls iNOS dimerization and substrate binding (Werner et al. 1998). iNOS enzyme activity in mouse

cells is maximal when iNOS binds BH_4 at a 1:1 ratio (Hevel and Marletta 1992). Importantly, BH_4 stabilizes the dimeric, active NOS enzyme (Giovanelli et al. 1991; Xie et al. 1996; Baek et al. 1993; Klatt et al. 1995). Crystal structures of murine iNOS oxygenase domains with or without BH_4 show that upon binding, BH_4 causes a conformational change in the NOS structure, forming a large dimer interface with a substrate channel and pterin binding sites (Crane et al. 1997, 1998). The large dimer interface contains more than 85 residues and contributes to the high stability of the iNOS dimer (Crane et al. 1998). BH_4 binding also enhances the affinity of iNOS for L-arginine, and L-arginine enhances BH_4 binding (Brand et al. 1995; Klatt et al. 1994). In addition to its allosteric effects on iNOS, BH_4 increases iNOS activity by preventing the direct binding of NO to the heme cofactor (Griscavage et al. 1994). BH_4 also scavenges free NO through reaction with O_2^- generated during BH_4 autoxidation, forming $ONOO^-$ in the process (Mayer et al. 1995). BH_4 is sensitive to oxidative stress, as ROS/RNS leads to the oxidation and conversion of BH_4 to quinonoid 5,6-dihydrobiopterin and finally to 7,8-dihydropterin, which is not a NOS cofactor (Milstien and Katusic 1999). The importance of BH_4 in iNOS stability and activity is highlighted by the fact that BH_4 deficiency in rodents results in altered NOS activity, in NOS uncoupling, and in the production of ROS/RNS (Brand et al. 1995; Cosentino et al. 1998).

iNOS activity is also controlled by the availability of its substrate L-arginine. In addition to iNOS, L-arginine is a substrate for arginase, the final enzyme of the urea cycle. Since both enzymes compete for L-arginine, controlled regulation of arginase expression or activity opposes NOS production of NO. iNOS and arginase are often reciprocally expressed in inflammatory responses (Mills et al. 2000; Mantovani et al. 2004). Incubation of macrophages with IFNγ and TNFα stimulates iNOS expression while suppressing arginase expression. Conversely, IL-4 and IL-10 upregulate arginase and suppress iNOS induction (Munder et al. 1999). In addition to limiting the synthesis of NO by iNOS, increased arginase expression can promote iNOS uncoupling, which leads to increased ROS production (Heusch et al. 2010; Wells and Holian 2007). The resultant increase in oxidative stress leads to further NOS uncoupling through the oxidation of BH_4 (Milstien and Katusic 1999).

8.3.5 Feedback Regulation of iNOS Expression and Activity by NO

NO-mediated modification of proteins by *S*-nitrosylation affects signaling pathways that control iNOS expression through feedback regulation. *S*-nitrosylation of IKKβ at Cys-178 can inhibit activation of the NFκB pathway. This posttranslational modification of IKKβ prevents its kinase activity, which is needed for phosphorylation and degradation of IκBα and downstream activation of NFκB that drives iNOS gene expression (Reynaert et al. 2004). In contrast, NO can augment iNOS gene expression through other mechanisms. Chemical NO donors increase

IL-1β-induced iNOS mRNA and protein expression and nitrite production in rat mesangial and aortic smooth muscle cells by increasing activation of NFκB binding to the iNOS promoter (Muhl and Pfeilschifter 1995; Boese et al. 1996; Lander et al. 1993). The effect of NO on the induction of iNOS transcription may be dose dependent. Low concentrations of the NO donor diethylamine dinitric oxide (DEA/NO) increase IFNγ/LPS-induced iNOS expression, while high doses of DEA/NO reduce it (Sheffler et al. 1995). In human mesangial cells, NO regulates iNOS expression in a biphasic manner, augmenting iNOS mRNA and protein expression at early time points, followed by an inhibitory effect on iNOS expression at later times (Perez-Sala et al. 2001). The mechanism by which this occurs may be attributed to opposing roles of NO on NFκB activity that manifest differently at early and late time points after cytokine induction (Diaz-Cazorla et al. 1999). Finally, endogenous production of NO that occurs as a result of the induction of iNOS expression amplifies iNOS protein levels through the *S*-nitrosylation of Ras, which drives the activation of this signaling protein. This results in the downstream activation of Akt and mTOR, which increases iNOS protein levels through a posttranscriptional mechanism (Lee and Choy 2013).

In addition to feedback mechanisms that control iNOS protein expression, NO can affect iNOS activity through multiple mechanisms. NO can compete with oxygen to bind the heme prosthetic group, forming a NO-heme complex that inhibits NOS activity (Mayer et al. 1995; Wink and Mitchell 1998). This gas can also increase arginase activity through *S*-nitrosylation, which inhibits NO production and NOS dimerization by limiting substrate availability (Wang et al. 1995; Santhanam et al. 2007; Kim et al. 2009b; Zunic et al. 2009; Heusch et al. 2010; Wells and Holian 2007). NO also directly *S*-nitrosylates iNOS, which disrupts dimerization of this enzyme (Mitchell et al. 2005). All NOS isoforms contain a zinc tetrathiolate (ZnS_4) cluster at the dimer interface, which contributes to dimer stability and is sensitive to regulation by NO (Miller et al. 1999; Mitchell et al. 2005). The formation of NOS dimers requires the coordinated interactions between one zinc atom and two cysteine residues from a CXXXXC motif in each NOS monomer (Miller et al. 1999). Treatment of purified, dimeric iNOS with the chemical NO donor DEA/NO leads to *S*-nitrosylation of tetrathiolate cysteines at positions 104 and 109 of the iNOS protein, reduces zinc binding by iNOS, and increases the level of inactive iNOS monomers. This indicates that *S*-nitrosylation of tetrathiolate cysteines disrupts their ability to bind zinc, leading to NOS uncoupling and loss of NOS activity (Mitchell et al. 2005). The importance of these cysteine residues in cell biology is supported by the fact that transfection of cells with iNOS proteins incapable of forming the tetrathiolate cluster results in reduced zinc binding by iNOS and drastically reduced production of NO (Mitchell et al. 2005). Similarly, mutations in C115 (crucial for zinc binding) in human iNOS abolish dimer formation and iNOS activity (Kolodziejski et al. 2003).

8.4 Conclusion

In the immune system, NO produced by iNOS is important for clearance of pathogens and regulation of adaptive immunity. The expression of iNOS is extensively controlled through transcriptional, translational, and posttranslational mechanisms. Appreciating these processes is essential for understanding immune function and dysfunction.

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Chapter 9
Role for Mechanotransduction in Macrophage and Dendritic Cell Immunobiology

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Abstract Tissue homeostasis is not only controlled by biochemical signals but also through mechanical forces that act on cells. Yet, while it has long been known that biochemical signals have profound effects on cell biology, the importance of mechanical forces has only been recognized much more recently. The types of mechanical stress that cells experience include stretch, compression, and shear stress, which are mainly induced by the extracellular matrix, cell–cell contacts, and fluid flow. Importantly, macroscale tissue deformation through stretch or compression also affects cellular function.

Immune cells such as macrophages and dendritic cells are present in almost all peripheral tissues, and monocytes populate the vasculature throughout the body. These cells are unique in the sense that they are subject to a large variety of different mechanical environments, and it is therefore not surprising that key immune effector functions are altered by mechanical stimuli. In this chapter, we describe the different types of mechanical signals that cells encounter within the body and review the current knowledge on the role of mechanical signals in regulating macrophage, monocyte, and dendritic cell function.

Cells in tissues are not just a collection of single independent units. They are continuously influenced by their environment as they interact with the surrounding extracellular matrix (ECM) through adhesion and remodeling and with close neighbor cells through cell–cell contacts and paracrine signaling. Furthermore, cells in distant tissues can influence each other through, for example, hormone signaling.

It is well accepted that biochemical signaling is an important route of cellular communication, regulating crucial biological processes such as proliferation, differentiation, migration, or apoptosis. By contrast, the importance of mechanical

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signals in controlling these processes has only been recognized much more recently. Yet, mechanical cues from neighboring cells, fluid flow, or the ECM have been shown to potentially be equally or, in some cases, more important than biochemical signals in determining particular cell fates. Furthermore, changes in the mechanical characteristics of tissues have been shown to play an important role in the progression of multiple pathologies like tissue fibrosis, atherosclerosis, and cancer. Unraveling the role of mechanical cues in specific cell types or tissues may therefore help to understand the true and complex interaction in the tissue microenvironment in vivo, which cannot merely be explained by biochemical signals alone.

In the following section, we will identify and describe the most important mechanical cues that cells can sense and highlight one by one their effect on cellular phenotype and function. Next, we will discuss how mechanical signals play a role in regulating immunological and cellular processes in monocytes, macrophages, and dendritic cells.

9.1 Mechanical Signals and Their Role in Cell Biology

Mechanical cues acting upon an object such as a cell are measured by the physical quantity stress, defined as force per unit area (pascal, Pa = N/m^2), and can be divided into three types: (1) compressive stress, stress acting perpendicular to the cell surface leading to compression; (2) elongation stress, stress acting perpendicular to the cell surface leading to extension; and (3) shear stress, stress acting parallel to the cell surface (Fig. 9.1a). When stress acts upon an object, the object can deform and the degree of deformation is measured by the quantity strain. Strain is calculated from changes in the dimensions of the object before and after application of the force (Janmey and Weitz 2004).

There are many ways in which the microenvironment can create stress on a cell (Fig. 9.1b). One of the most important microenvironmental factors is the ECM. Mechanical properties of the ECM such as stiffness and topography have been shown to control differentiation, proliferation, and function in a large variety of cell types, and ECM substrate availability, for example, has been shown to control cell shape, resulting in alterations in cell viability or cellular functions, such as migration (Chen et al. 1997; Brock et al. 2003). Another important microenvironmental factor that generates stress is fluid flow. Fluid flow in the vasculature or the lymph vessels generates shear stress in blood and lymphatic endothelial cells, respectively, and cellular alignment to fluid flow and inflammatory processes have been shown to be particularly regulated by fluid shear stress in these cells (Wang et al. 2013a). Mechanical stress can also be generated through cell–cell adhesion contacts, and, as such, neighboring cells receive mechanical signals that are transmitted through intercellular cytoskeletal connections. Finally, cells can encounter mechanical stress as a consequence of forces exerted on a macroscale. Cells in organs that

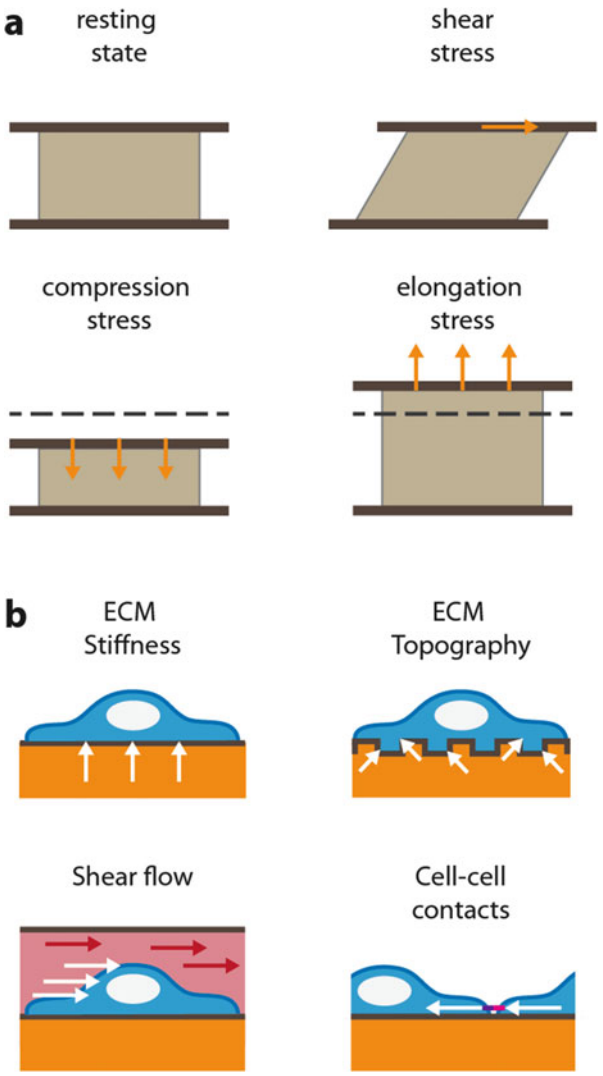

Fig. 9.1 Types of mechanical stress and mechanical cues affecting cells. (**a**) Objects can experience three types of stress, depending on the direction of the force compared to the surface on which the object rests: force parallel to the surface leading to shear stress, force perpendicular to the surface leading to compression stress (directed toward the surface), or elongation stress (directed away from the surface). (**b**) Examples of mechanical cues sensed by cells: properties of the extracellular matrix, like stiffness or topography, can affect cell function. In the bloodstream, blood flow leads to shear stress in cells. Also, cell-to-cell contacts can transduce mechanical cues from one cell to the other

undergo mechanical loading, such as bone or cartilage tissue, or experience stretch, like the skin or the lungs, can be crucially affected by these mechanical signals.

It is important to note that, next to external mechanical stress, cells themselves do not display a constant mechanical state. The cytoskeleton, which provides structure to the cell, is highly dynamic, and remodeling of actin polymers and microtubules provides the cell with a mechanism to internally generate forces and change its own mechanical characteristics. In this way, cells do not simply feel mechanical perturbations, but can adjust their response to these signals by changing their cytoskeleton or their subcellular structures adhering to the substrate (Guilak et al. 2009).

9.1.1 ECM Stiffness

Stiffness is defined as the resistance of an object to deformation, typically measured in newtons per meter. In contrast, "elasticity" or "elastic modulus" is a characteristic of the object's material, defined as the ratio of stress and strain, measured in newtons per square meter (pascal). Importantly, while elasticity is not affected by the object's dimensions, stiffness is. Still, stiffness depends on the elasticity of the material: the higher the elastic modulus of an object's material, the higher its stiffness. Confusingly, both stiffness and elasticity are interchangeably used in studies investigating the effect of ECM stiffness, but the unit used to mechanically describe model substrates in these studies is pascal, thereby referring to elasticity of the material.

Within the body, tissues exhibit a broad stiffness range from ~0.1 kPa for very soft tissues like the brain, ~3 kPa in stromal tissues, and ~12 kPa for skeletal muscle and cartilage to >100 kPa for bone (Cox and Erler 2011). Importantly, under pathological conditions, tissue stiffness can be different. Tumors, for example, often have an increased stiffness compared to healthy tissue (Butcher et al. 2009), and also in fibrotic or atherosclerotic lesions, tissues often stiffen (Georges et al. 2007; Huynh et al. 2011). For renal disease models, however, it has been shown that the renal glomerular tissue decreases in stiffness (Wyss et al. 2011). It is therefore not surprising that effects of ECM stiffness on cell phenotype and function are the most studied characteristic in mechanobiology. ECM stiffness is, for example, studied in stem cells, since understanding the role of substrate stiffness in sustaining or differentiating stem cell function ex vivo is key to engineering in vitro tissues for in vivo applications. In this regard, Engler and colleagues were the first to show that ECM stiffness can direct stem cells toward specific cell lineages (Engler et al. 2006). Human mesenchymal stem cells (MSCs) were placed on polyacrylamide gels coated with collagen type I with varying stiffness and cultured for a week. Cells cultured on soft matrices (0.1–1 kPa), matching brain tissue stiffness, exhibited a typical neuronal phenotype. Cells on intermediate-stiffness matrices (8–17 kPa), matching skeletal muscle tissue, adapted to a myogenic lineage, and cells on stiff matrices (25–40 kPa) underwent osteogenesis. This stiffness-directed lineage specification was shown to be dependent on non-muscle myosin-II function and has been further substantiated by multiple studies, also in 3D (Her et al. 2013; Jaramillo et al. 2015) using stem cells derived from different sources such as the umbilical cord (Witkowska-Zimny et al. 2012), amniotic fluid (Skardal et al. 2013), embryo (Evans et al. 2009), and bone marrow (Winer et al. 2009). Importantly, the extent of stiffness-directed lineage commitment greatly depends on the differentiation stage of the cell (Hsiong et al. 2008; Witkowska-Zimny et al. 2013). Furthermore, Choi et al. demonstrated that soft substrates of 0.1 kPa could direct reprogramming of differentiated mouse embryonic fibroblasts to induced pluripotent stem cells (Choi et al. 2016). Also, it has been shown that stem cells on substrates with stiffness below 0.25 kPa display cell cycle arrest, but maintain their stemness (Winer et al. 2009; Chowdhury et al. 2010). In addition, when

mechanical stiffness changes at a high frequency, MSCs display the same kind of self-sustaining behavior (Frank et al. 2016). Overall, these studies indicate that very soft ECM stiffness can maintain stemness in stem cells, while stiffer substrates drive stem cells toward specific lineages. It is, however, important to note that only in concert with biochemical cues from the tissue microenvironment irreversible commitment toward a specific lineage can be induced in stem cells.

Not only pluripotent stem cells but also a wide spectrum of terminally differentiated cells have been shown to be affected by substrate stiffness. Among others, fibroblasts (Lo et al. 2000; Yeung et al. 2005), neurons (Flanagan et al. 2002), osteoblasts (Khatiwala et al. 2006), muscle cells (Engler et al. 2004; Peyton and Putnam 2005), cardiomyocytes (Engler et al. 2008), endothelial cells (Deroanne et al. 2001), chondrocytes (Chen et al. 2014), epithelial cells (Eisenberg et al. 2011; Wang et al. 2012), beta cells in the pancreas (Naujok et al. 2014), endometrial cells (Matsuzaki et al. 2016), and hepatocytes (Semler et al. 2005; Kourouklis et al. 2016) have all been shown to be affected by substrate stiffness. Substrate stiffness can affect a cell's ability to proliferate, migrate, differentiate, or go into apoptosis (Pelham and Wang 1997; Wang et al. 2000; Lo et al. 2000; Saez et al. 2007), as well as processes like phagocytosis (Boochoon et al. 2014) and endocytosis (Brugnano and Panitch 2014). Endothelial cells (ECs) and epithelial cells have been shown to organize into three-dimensional structures when cultured on substrates with low stiffness, which was proposed to be due to cell–cell adhesions being dominant over cell–matrix adhesions on soft substrates (Deroanne et al. 2001; Shebanova and Hammer 2012).

Stiffness is an important factor in tissue homeostasis, and changes in stiffness play an important role in pathological processes. For example, cells that are seeded on a substrate that is much stiffer than the tissue they originate from often display fibrosis-related phenotypes, indicating a direct role for tissue stiffness in disease progression (Liu et al. 2010; Olsen et al. 2011; Shi et al. 2013; Xie et al. 2014). Also in cancer, tissue stiffness plays an important role. In breast cancer, tumor tissue is often noticed as stiffened tissue clumps present in the breast tissue, and increasing tumor stiffness correlates with invasiveness of the breast cancer type (Chang et al. 2013). Mechanistically, it has been shown that breast cells in a stiff matrix display altered contractility and adhesion to the matrix, leading to integrin clustering and the activation of several pathways that drive progression of the cells toward a more malignant phenotype (Paszek et al. 2005). Levental and colleagues found that the main determinant for the effect of tissue stiffening on cancer cell progression and invasion is collagen cross-linking within the ECM. They identified lysyl oxidase (LOX), a collagen cross-linking enzyme also involved in fibrosis, as a crucial player in this effect, as inhibition of LOX prevented fibrosis and reduced tumor incidence (Levental et al. 2009). Not only breast cancer cells sense and respond to ECM stiffness. Several other different types of cancer cells have been shown to alter their responsiveness to drugs (Schrader et al. 2011; Zustiak et al. 2014), proliferation (Grundy et al. 2016), or metastatic ability (Tang et al. 2010) in response to a changed ECM stiffness.

Concluding, it is clear that the stiffness of the surrounding ECM is an important mechanical cue affecting cells, regardless of which tissue they inhabit. In the

stiffness range of healthy tissues, ECM stiffness can direct physiological processes like differentiation, proliferation, and migration. However, when ECM stiffness is increased beyond the physiological range, cells inhabiting this stiffened tissue can experience abnormal signaling and adhesion, potentially leading to a malignant phenotype or even tumor progression.

9.1.2 ECM Topography

In the context of mechanical cues, the term topography refers to the physical configuration of the surface of the ECM/substrate, including its relief, its features, and their specific geometry. Surface topography has been shown to be very important in cellular response to engraftment of implants. Cells important in this process, such as osteoblasts, osteoclasts, and fibroblasts, can all sense topographical cues like present pits or the roughness of the surface (Berry et al. 2004; Ball et al. 2008; Geblinger et al. 2010). In addition, topography can influence a wide variety of cell types, including MSCs (Kulangara et al. 2014; Abagnale et al. 2015), epithelial cells (Kim et al. 2014), neurons (Micholt et al. 2013; Li et al. 2015b), muscle cells (Bajaj et al. 2011), ECs (Dreier et al. 2013), and cancer cells (Tan et al. 2015; Chaudhuri et al. 2016). Cells typically align along micropatterns, (Martinez et al. 2009) and data from many studies suggest that cells present a more "in vivo-like" phenotype on surfaces with micropatterns compared to flat surfaces (McKee et al. 2012; Yang et al. 2014). Topographical cues have been shown to affect cell morphology, adhesion (Stanton et al. 2015), stem cell differentiation, and endocytosis, among others (Teo et al. 2011; Huang et al. 2016), but also, for example, collective cell migration seems to be affected by substrate topography (Doxzen et al. 2013). Finally, it has been shown that the wound healing rate of corneal epithelial cells was increased by topographical patterns (Yanez-Soto et al. 2013).

Interestingly, in some studies the role of both substrate topography and stiffness has been investigated. Chaterji and colleagues studied vascular smooth muscle cells (vSMCs) on substrates with two different stiffnesses, either patterned or unpatterned. They showed that topography and stiffness had a synergistic effect, creating a pathological hypercontractile vSMC phenotype on the stiff patterned substrate and a healthy contractile phenotype on the soft patterned substrate (Chaterji et al. 2014). vSMCs on unpatterned substrates, both stiff and soft, showed a more synthetic phenotype. Lu and colleagues investigated the response of mouse embryonic stem cells to substrate stiffness and topography by culturing them on planar, grooved, hexagon, or pillar substrates with two different stiffnesses (Lu et al. 2014). They found that topography seems to be more effective on stiff substrates in retaining stemness in these cells. Yang and colleagues created substrates with a controllable size of stiff or soft regions and studied the behavior of MSCs on these substrates (Yang et al. 2016). Cells on substrates with higher concentration of stiff regions displayed a spread, elongated morphology and expressed osteogenic markers. However, when the spatial organization of these

stiffer regions was altered from a regular to a randomized pattern, cellular response was changed, with morphologies closer to those of cells on soft substrates and higher expression of stem cell markers.

In conclusion, ECM topography, as a separate mechanical cue or together with ECM stiffness, can have a major influence on cell phenotype and function in a wide variety of cell types.

9.1.3 Cell Shape

Changes in ECM stiffness and topography alter cellular shape, which in turn affects the cell's mechanical properties. However, it has been shown that differences in cell shape per se can also impact cells. Altering cell shape is accompanied by changes in the cell's cytoskeleton. Already in 1997 Chen et al. demonstrated that spreading of ECs affects their growth and apoptosis and that this effect did not depend on the contact area between cell and ECM, but on the shape of the cell itself (Chen et al. 1997). Later, it was shown that cell shape can control cell stiffness (Thery et al. 2006; Tee et al. 2011), adhesion (Chen et al. 2003), and directed migration (Parker et al. 2002; Brock et al. 2003).

MSCs are frequently used to study manipulations in cell shape (Kilian et al. 2010; Peng et al. 2011). Yang and colleagues cultured MSCs derived from different organs on substrates coated with membranes containing either 10 or 20 μm diameter holes and showed that only cells on the larger islands elongated and differentiated into smooth muscle cells, irrespective of their origin tissue (Yang et al. 1999). McBeath et al. investigated effects of cell shape on MSC lineage commitment by growing cells on fibronectin islands of different sizes in a mixture of adipogenic and osteogenic induction media (McBeath et al. 2004). They showed that human MSCs cultured on small islands, exhibiting a round shape, differentiated into adipocytes, whereas cells grown on large islands had a spread-out shape and expressed osteogenic markers. Moreover, studying both ECM stiffness and cell geometry, it has been shown that altering cell shape by specific substrate geometries resulted in elevated osteogenesis in MSCs compared to cells on unpatterned substrates (Lee et al. 2014).

Overall, cellular shape has a major influence on basic cell properties such as adhesion, growth, and apoptosis, and, importantly, effects on cell properties exerted by ECM stiffness or topography may therefore be (partly) explained by changes in cell shape.

9.1.4 Flow

Flow is the continuous and directed movement of a fluid. Examples of flow in the body include vascular flow, interstitial flow, lymphatic flow, and urinary flow. ECs, which line the interior surface of all blood and lymphatic vessels, continuously

undergo fluid shear stress imposed by flow and are by far the most studied cell type with respect to fluid shear stress (Olivier et al. 1999). Fluid shear stress affects EC mechanical properties, and ECs sense the direction of flow, since they can align accordingly (Mathur et al. 2007; Wang et al. 2013a). Interestingly, while undisturbed laminar flow is thought to be atheroprotective, disturbed flow creates an altered microenvironment permissive to atherosclerosis (Niwa et al. 2004; Heo et al. 2014). ECs can have different responses to flow depending on the specific tissue in which they are located and the type of vessel they line (blood or lymphatic) (Ng et al. 2004; Siddharthan et al. 2007). Flow also critically controls endothelial sprouting in angiogenesis, an important process in many (patho)physiological conditions such as wound healing or tumor growth. Shear flow parallel to the endothelial surface has been shown to inhibit EC sprouting, whereas interstitial flow from extravasating plasma and transmural flow perpendicular to the EC surface trigger and maintain EC sprouting (Song and Munn 2011; Galie et al. 2014). ECs respond differentially to flow on substrates with different stiffnesses (Galie et al. 2015) indicating that vascular stiffening with age or upon disease can further implicate vessels for vascular pathology. Furthermore, shear flow can prevent apoptosis of ECs on micropatterns with anisotropic geometry (Wu et al. 2007). However, the direction of the flow with respect to the anisotropic patterns is important: flow perpendicular to the pattern induced an opposing effect to topography, whereas topography and parallel flow showed synergistic effects (Morgan et al. 2012).

Besides having an effect on ECs, fluid shear stress also plays a role in lymphocyte rolling and adherence to the vessel wall (Campbell et al. 1998). Furthermore, fluid shear stress can affect MSC differentiation in bone marrow niches (Sonam et al. 2016) and can induce platelet production by megakaryocytes (Jiang et al. 2014; Luff and Papoutsakis 2016).

Also cancer cells present in the vasculature are affected by flow. It has been shown that tumor cells subjected to fluid shear stress undergo cell cycle arrest (Chang et al. 2008). Furthermore, in a microfluidic system, cell viability of circulating cancer cells was shown to be decreased upon circulation time. However, under physiologically relevant flow, surviving cancer cells retained their ability to proliferate (Fan et al. 2016). This suggests that cancer cells change to a quiescent state in the circulation, whereas they reactivate their proliferative activity after having left the circulation. Flow has also an impact on cancer cell extravasation: exposure to shear flow induces expression of adhesion proteins allowing cancer cell adhesion to vascular endothelium (Liu et al. 2009; Zhao et al. 2014) and production of matrix degrading enzymes needed for extravasation (Wang et al. 2015). In addition, studies have indicated that shear flow is also involved in formation of leukocyte–cancer cell aggregates that facilitate cancer cell extravasation (Jadhav et al. 2001; Liang et al. 2008).

Thus, changes in the dynamics of fluid flows, which alter fluid shear stress on cells, can have major impact on (patho)physiology, highlighting the importance of this mechanical cue in biology.

9.1.5 Mechanical Stretch or Compression

Stretch is the deformation of an object subjected to elongation stress, and compression is the deformation due to compression stress. Stretch and compression can be caused by stress that is applied to an object continuously, but also cyclically.

An organ for which stretch and subsequent expansion of the tissue is crucial for its function is the lung. Cyclic expansion of the lung not only results in continuous exchange of oxygen-rich and oxygen-low air. This ventilation also results in stretch of lung epithelial cells, which are heavily influenced by stretch: viability of and surfactant secretion by alveolar epithelial cells are both strongly affected by stretch (Edwards et al. 1999; Arold et al. 2009). Also in the context of mechanical ventilation-related injury, stretch on lung cell types has been investigated. Reactive oxygen species generation, intracellular glutathione and cytokine production, all important in inflammatory responses, were altered in alveolar cells upon stretch (Jafari et al. 2004; Chapman et al. 2005; Hammerschmidt et al. 2005). In addition, secretion or expression of enzymes involved in ECM remodeling such as matrix metalloproteinase 2 (MMP-2) and MMP-7 are shown to be affected by stretch (Patel and Kwon 2009; Yerrapureddy et al. 2010).

Another type of tissue that is highly subjected to stretch and compression is bone (Ehrlich and Lanyon 2002). Mechanical loading in bone induces bone formation, initiated by mechanosensing osteocytes (Nomura and Takano-Yamamoto 2000; Reijnders et al. 2007). Bone-derived MSCs that are subjected to cyclic stretch have been shown to favor osteoblastogenesis above adipogenesis, providing an explanation for the increase in bone formation upon mechanical loading (David et al. 2007). Likewise, osteoclasts, the bone-resorbing cells, are sensitive to stretch (Li et al. 2015a), and mechanical cues can affect osteocyte–osteoclast communication in inflammatory settings such as rheumatoid arthritis, potentially preventing osteoporosis (Pathak et al. 2015).

Cells in cartilage are also subjected to mechanical loading, and cartilage matrix production can be stimulated by dynamic compression (Grodzinsky et al. 2000). In addition, local MSCs can be directed toward chondrogenesis upon cyclic compression (Angele et al. 2003; Huang et al. 2004; Terraciano et al. 2007), while the frequency and duration of the cyclic loading modulates this differentiation into chondrocytes (Elder et al. 2001).

In addition to the lung, bone, and cartilage, multiple other organs undergo stretch or compression, and cells originating from these tissues have all been shown to respond to stretch or compression in vitro. Among others, cardiomyocytes (Mihic et al. 2014; Banerjee et al. 2015), differentiating myoblasts (Soltow et al. 2013; Chang et al. 2016), vSMCs and ECs (Wilkins et al. 2014; Wanjare et al. 2015; Tian et al. 2016), bladder smooth muscle cells (Kushida et al. 2016), and endometrial stromal cells in the uterus (Izumi et al. 2015) have been shown to respond to stretch or compression.

In conclusion, cells are not only sensitive to static mechanical cues like ECM stiffness and topography, but are also heavily influenced by dynamic mechanical

cues such as stretch or compression. Important in the context of these mechanical signals are the directions of stretch or compression (uniaxial, biaxial, or isotropic), the frequency, and the magnitude of the stress applied.

9.2 Mechanical Signals in Macrophages, Monocytes, and Dendritic Cells

Cells from the immune system are present in tissues throughout the body, contributing to the host's defense mechanism against pathogens and nascent tumor cells. Within these very diverse tissue locations, immune cells can experience diverse mechanical environments. Moreover, some immune cells, such as dendritic cells (DCs) or T cells, migrate from one tissue to the other, experiencing multiple mechanical signals during their lifetime. As such, one can imagine that mechanical signals, by modifying the function of immune cells, can have profound effects on immunity.

Macrophages are mononuclear phagocytic cells that are part of the innate immune system. They are tissue-resident immune cells that are locally involved in engulfment and early clearance of pathogens, as well as tissue remodeling and repair in wounded tissues (Haniffa et al. 2015). Macrophages in tissues already develop in the embryo, before final stage of embryonic hematopoiesis, so-called "definitive" hematopoiesis (Ginhoux and Guilliams 2016). Since tissue-resident macrophages are already present in a specific tissue microenvironment in the embryo, they develop along with their specific tissue (Mass et al. 2016). Indeed, it has been shown that macrophages isolated from different locations behave differently (Wang et al. 2013b). Each tissue therefore has its own macrophage population adapted to the local functional needs of the microenvironment.

With respect to function and development, macrophages are very closely related to monocytes and DCs (Haniffa et al. 2015). Monocytes and DCs are derived from a common progenitor in hematopoietic stem cells in the bone marrow. This progenitor, named monocyte/macrophage and dendritic cell progenitor (MDP), can give rise to the common monocyte precursor (cMoP), which generates monocytes, or give rise to the common dendritic cell progenitor (CDP) which generates DCs (Hettinger et al. 2013; Naik et al. 2007; Onai et al. 2007). Monocytes can be divided into two subsets that are involved in either protection against infections or wound healing and repair (Serbina et al. 2008; Thomas et al. 2015). Two different DC subsets develop from the CDP: plasmacytoid DCs (pDCs) which fully differentiate in the bone marrow and classical DCs (cDCs) which leave the bone marrow in a pre-cDC state and fulfill differentiation in the peripheral tissues (Satpathy et al. 2012).

The fact that mechanical signals can have a crucial impact on the immune system is nicely illustrated in two studies on embryonic hematopoiesis. These studies independently identified blood flow-induced shear stress as a determining factor in the embryonic hematopoiesis process. Using a zebra fish model, North and

colleagues demonstrated that blood flow is crucial for definitive hematopoiesis. The effect of blood flow on development of hematopoietic stem cells (HSCs) was mediated by nitric oxide (NO), synthesized by HSCs themselves. A similar effect on HSCs was found in mice, where knockout of NO synthase or treatment of the mother with an NO inhibitor led to a hematopoietic cluster decrease or absence in the embryo, respectively (North et al. 2009). The importance of flow in hematopoiesis was further substantiated by Adamo et al., who showed that hematopoietic commitment was induced by flow-induced shear stress in mouse embryonic stem cells and embryonic cells derived from the aorta–gonad–mesonephros region, the tissue in the embryo where HSC progenitors originate. Also in this study, the flow-induced effect on hematopoiesis was found to be regulated by NO (Adamo et al. 2009).

Within peripheral tissues, macrophages, as well as monocytes and DCs, can experience any of the major mechanical signals like ECM stiffness, topography, compression, or stretch. However, it depends on the specific tissue to what level this mechanical cue plays a role: macrophages and DCs in the lungs are intensively subjected to cyclic stretch, but stretch does not play a role in macrophage-like microglia cells in the brain, where most likely the low ECM stiffness determines cell function.

Upon generation in the bone marrow, monocytes and (pre-)DCs enter the blood circulation. Within the vasculature, blood flow and associated shear stress can critically affect these immune cells in their survival, phenotype, or activity. To reach peripheral tissues, monocytes and DCs first need to cross the vessel wall through a process of transendothelial migration. Initial adherence to the vessel wall is important for this process, and also here, blood flow plays an important role. In addition to the blood vasculature and peripheral tissues, DCs are also present in the lymphatics. As specialized antigen-presenting cells (APCs), upon recognition and uptake of an antigen, DCs travel from peripheral tissues through the lymph vessels to the lymph node. Here they activate antigen-specific T cells, leading to either tolerance or an adaptive immune response. The lymphatics and lymph node are yet additional distinct mechanical environments for DCs, where local flow and extracellular pressure can affect DC function.

Mononuclear phagocytic cells do not only encounter mechanical cues of the physiological environment but are also often the first cells to reach sites of implantation. As such, macrophages and DCs come into contact with implants made of diverse biocompatible materials, each with their own stiffness and topography. Also, blood monocytes interact with implant surfaces since surgical implantation leads to local tissue damage, and blood can collect in the space between implant and tissue. This so-called foreign body response of monocytes, macrophages, and DCs is crucial in local engraftment of the implant and can determine whether the implant is rejected by the body or not.

In the following paragraphs, we will review the current knowledge on mechanobiology in monocytes, macrophages, and DCs and discuss how these cells are affected in their phenotype and function by different mechanical cues.

9.2.1 Monocytes

9.2.1.1 Monocyte Response to Blood Shear Flow

Blood circulation is the primary site where monocytes are present, and mechanical stress through blood flow exposure has a major impact on monocyte phenotype and function. Indeed, already in the 1990s, Pritchard and colleagues identified fluid circulation and subsequent shear stress as determining factors for the location in the vasculature of circulating monocytes (Pritchard et al. 1995). In this way, flow by itself can be a determining factor for location of monocyte-rich early atherosclerotic regions in the vessel wall.

In vitro investigation of locomotion of different subsets of monocytes under shear flow conditions has indicated that subsets display differences in locomotory behavior: nonclassical monocytes prefer to adhere to the endothelium, whereas classical monocytes display more migratory, long-range crawling behavior (Collison et al. 2015). Different types of shear flow may therefore lead to differential migratory activity in monocyte subsets, correlating to their distinct functions.

When migrating to peripheral tissues, monocytes need to leave the vasculature, which occurs through the process of transendothelial migration. Adhesion of monocytes to ECs is very important in this process, and this monocyte–EC interaction is heavily influenced by flow. Indeed, it has been shown that human primary monocytes cultured ex vivo rapidly lose their transendothelial migration ability; when exposed to shear flow during culture, this ability can be maintained (Tsubota et al. 2014). Dynamics in fluid shear stress regulate expression of adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-/P-selectin on EC surfaces, modulating monocyte adhesion to ECs and subsequent extravasation (Cicha et al. 2008).

Some intracellular pathogens can use monocytes as a vehicle for dissemination throughout the body. Evidence emerging from multiple studies indicates that mechanical factors may play a role in pathophysiology of these infections. THP-1 monocytes infected with *Chlamydia muridarum* showed increased pro-inflammatory cytokine production, increased expression of adhesion molecules, and subsequently increased adhesion to ECs under shear compared to static conditions (Evani et al. 2011). Similarly, THP-1 monocytes infected with *Chlamydia pneumoniae* produced more interleukin-1β (IL-1β) and more chemokines under shear compared to static conditions. (Evani et al. 2013; Cheeniyil et al. 2015). *Toxoplasma gondii*-infected monocytes display an increased transendothelial migration compared to uninfected monocytes. Subjecting them to shear flow even increased this migration more than fourfold compared to static conditions (Ueno et al. 2014). These data nicely illustrate that mechanical cues like shear flow do not only have a role in normal physiology but can also contribute to the development of pathophysiology, like infectious diseases.

9.2.1.2 Monocyte Response to Stretch and Substrate Stiffness

In some parts of the vasculature, the vessel wall undergoes pulsed stretch due to strong and pulsatory flow. Yun and colleagues showed that stretch of ECs influences monocyte adhesion to the vessel wall. EC monolayers were subjected to different extents of stretch at multiple stretch rates. Human primary monocytes were left to adhere to this stretched monolayer. At different stretch rates, monocyte adhesion was increased when ECs experienced larger stretch. This effect was observable even after 24 h (Yun et al. 1999).

Also substrate stiffness has been shown to modify monocyte adhesion to ECs. MacKay and Hammer demonstrated that in shear flow conditions, THP-1 monocytic cells increasingly adhered to E-selectin-coated gels in a stiffness-dependent manner (MacKay and Hammer 2016). Interestingly, Scott and colleagues identified a biphasic effect of ECM stiffness (Scott et al. 2016). While U937 monocytes displayed very low adhesion to ECs seeded on a gel of 1 kPa, adhesion to ECs on softer or stiffer gels was higher. Although in the Scott et al. study cells were not subjected to shear flow, it may in fact be that the study by McKay and Hammer has missed this biphasic effect of matrix stiffness on monocyte–EC adhesion, since the softest gel used in their study was 1 kPa.

9.2.1.3 Monocyte Response to Surgical Implants

A few studies have investigated the effects of mechanical cues on the monocyte response to clinically applied biocompatible materials. Eriksson et al. investigated the interaction of human whole blood with titanium surfaces with different topographies and found that upon long incubation times, monocytes adhered better to roughened surfaces than to smooth surfaces, regardless of the surface chemistry (Eriksson et al. 2001). In biocompatible scaffolds of electrospun polycaprolactone (PCL) nanofibers, monocytes showed significant lower binding to scaffolds with aligned fibers compared to scaffolds with randomly oriented fibers (Cao et al. 2010).

Shive and colleagues investigated monocyte apoptosis in the context of cardiovascular device-related infections. Primary human monocytes were left to adhere to polyurethane substrates while under shear stress (Shive et al. 2002), and the results indicated that monocytes underwent apoptosis in a shear stress-dependent manner, with significant monocyte apoptosis already after 4 h. High shear stress at the implantation site may therefore have negative effects on monocyte viability and subsequent clearance of implant-related infections.

Stents placed to treat vessel occlusion due to coronary atherosclerosis can experience reocclusion initiated by local inflammation. Investigating the response of THP-1 monocytes to stainless steel under shear stress, the group of Messer found that activated THP-1 monocytes on stainless steel under shear show elevated

cytokine production (Messer et al. 2008). This puts primary human monocytes forward as potential players in stent restenosis and stent function failure.

9.2.2 Macrophages

9.2.2.1 Macrophage Response to ECM/Substrate Stiffness

In multiple studies it has been established that ECM/substrate stiffness affects macrophage activation phenotype and function. Féréol and colleagues showed that alveolar macrophages alter their shape and intrinsic stiffness in response to substrate stiffness. This occurs through changes in both the cortical and deep cytoskeleton and, surprisingly, was claimed to be independent of stress fiber formation (Fereol et al. 2006, 2008). Substrate stiffness also determines the magnitude of the force generated in the leading edge of a macrophage, a process that is crucial for macrophage motility and mediated by the signaling axis involving Rac GTPase, Rho-associated protein kinase (ROCK), myosin-II, and PI3 kinase (Hind et al. 2015). For monocyte-derived macrophages seeded on polyacrylamide gels, it was found that they displayed increased cell area, increased proliferation, and higher migration speed on stiffer substrates (Adlerz et al. 2016). Interestingly, Previtera et al. also saw that unstimulated murine bone marrow-derived macrophages (BMDMs) had a higher velocity on the stiffer substrate, but when stimulated with lipopolysaccharide (LPS), the effect was reversed, and velocity was even lower on the stiff substrate compared to unstimulated macrophages on soft substrates (Previtera et al. 2015), suggesting that macrophage activation may influence the stiffness-mediated effects on migration. The authors proposed that unstimulated macrophages are always mobile, regardless of the tissue physical environment, whereas stimulated macrophages will move in soft healthy tissues but, once localized to a stiff region of tissue damage, will halt and perform their tasks in recovering the damaged tissue.

On biocompatible polyethylene glycol (PEG) hydrogels of varying stiffness, Blakney et al. found murine BMDMs to display a more spread morphology on stiffer substrates (Blakney et al. 2012). When stimulated with LPS, BMDMs displayed a classical M1 activation phenotype. The degree of activation however was lowest on the softest gels. Upon in vivo implantation of PEG scaffolds, the number of macrophages present at the implantation site was lowest for the softest gels 28 days post-implantation. This reduced macrophage activation and less severe in vivo host response therefore designated hydrogels with lower stiffness as more suitable options for in vivo tissue engineering (Blakney et al. 2012).

As macrophages on substrates with different stiffnesses show distinct morphologies, one way in which ECM stiffness might affect macrophage activation and phenotype might be through alterations in cell shape. Indeed, by solely changing cell shape, McWhorther and colleagues showed that murine BMDMs can be driven toward an M2 immunoregulatory phenotype (by elongated shape) or an M1 phenotype

(by rounded shape). Moreover, elongation enhances the effects of M2-inducing factors and counteracts effects of M1-inducing factors (McWhorter et al. 2013).

Irwin et al. used polyacrylamide–PEG hydrogels with different stiffnesses and found for THP-1 macrophages differentiated with phorbol 12-myristate 13-acetate (PMA) that adhesion increased with increasing substrate stiffness. However, when looking at pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) production decreased with increasing substrate stiffness, and interleukin-8 (IL-8) production showed a biphasic response (Irwin et al. 2008). Patel and colleagues also found that cellular stiffness increased with increasing substrate stiffness. Using RAW264.7 and U937 macrophage cell lines stimulated with LPS, they found that TNF-α production was influenced by substrate stiffness such that, at a higher stiffness, less TNF-α was produced, which is in agreement with Irwin et al. (Patel et al. 2012). In contrast, Previtera and Sengupta found that BMDMs produced more cytokines (among which TNF-α) on stiffer substrates in both non-stimulated and LPS-stimulated situations. Also, they showed that this effect was mediated through pattern recognition receptor Toll-like receptor 4 (TLR4), as $TLR4^{-/-}$ macrophages showed decreased cytokine production in response to substrate stiffness compared to wild-type macrophages. Furthermore, expression of the TLR4 adaptor protein MYD88 was increased in macrophages on stiffer substrates. Also, these macrophages displayed higher IκB protein phosphorylation and increased translocation of transcription factor NfκB on stiffer substrates (Previtera and Sengupta 2015).

Phagocytosis is a crucial activity performed by macrophages to fulfill their role in clearance of infection and tissue remodeling. The effect of substrate stiffness on macrophage phagocytosis has therefore also been assessed in many studies. Patel and colleagues found that both RAW 264.7 macrophages and human alveolar macrophages showed increased phagocytosis of beads on a stiffer substrate. In addition, when RAW macrophages were subjected to 10% isotropic stretch, phagocytosis was lower compared to unstretched cells, on both soft and stiff substrates (Patel et al. 2012). However, in the study of Adlerz and colleagues, phagocytosis of fluorescent beads by monocyte-derived macrophages was not affected by substrate stiffness (Adlerz et al. 2016). In contrast, Scheraga and colleagues found a stiffness-dependent effect on phagocytosis in LPS-stimulated murine BMDMs and alveolar macrophages. They identified transient receptor potential vanilloid 4 (TRPV4), a mechanosensitive ion channel, to be a major regulator in this stiffness-dependent effect (Scheraga et al. 2016).

Substrate stiffness thus clearly influences macrophage activation, cytokine production, and phagocytosis, but studies often do not agree on the extent of the effect and the exact relationship between ECM stiffness and macrophage functionality.

9.2.2.2 Macrophage Response to Compression

Compression of cells can be achieved by applying extracellular pressure to the cells. The group of Shiratsuchi and Basson has studied the effect of this type of compression on macrophages. They found that PMA-differentiated THP-1

macrophages display an increase in phagocytosis with increasing pressure (+20 and +100 mmHg). This effect was shown to be caused by focal adhesion kinase (FAK) activity which led to a decrease in activation of extracellular signal-related kinase (ERK) (Shiratsuchi and Basson 2004). In another study, they showed that this effect of increasing phagocytosis with increasing extracellular pressure can also be observed in primary human monocytes and monocyte-derived macrophages and identified p38 mitogen-activated protein kinase (MAPK) as an additional player involved in the effect of extracellular pressure on macrophage phagocytosis (Shiratsuchi and Basson 2005).

These data suggest that in tissues with increased pressure, like inflamed tissues, macrophages may phagocytose more efficiently to clean up the damage.

9.2.2.3 Mechanical Properties and Phagocytosis Targets

Not only the mechanical characteristics of the extracellular environment can affect macrophage phagocytosis, but also the mechanical properties of the target can modulate its own uptake. Beningo and Wang found that BMDMs engulfed more stiff particles compared to soft particles with the same chemistry. This was dependent on a Rac1 GTPase mechanosensory mechanism (Beningo and Wang 2002). Champion and colleagues identified particle shape, and not size, as a dominant factor in phagocytosis by alveolar macrophages. The authors suggested that the complexity of the actin structure necessary to initiate engulfment was determined by the local particle shape at the phagocytosis initiation site and that macrophages would only spread on the targets without internalizing them in the case this actin structure could not be created (Champion and Mitragotri 2006). Differences in phagocytosis and subsequent cytokine production due to particle shape were also found by other studies (Herd et al. 2013; Chen et al. 2016). Finally, also the topography of the particle to be ingested affects phagocytosis and macrophage activation. IL-1β secretion upon NLRP3 inflammasome activation was shown to be higher upon uptake of texturized particles than upon uptake of particles with a smooth surface (Vaine et al. 2013; Herd et al. 2013).

9.2.2.4 Macrophage Response to Stretch

A major tissue site where stretch can be experienced by cells is the lungs. In a mechanical ventilation model, Pugin and colleagues showed that human alveolar macrophages displayed an increased IL-8, IL-6, and TNF-α production upon stretch. For some cytokines, LPS stimulation and stretch showed a synergistic effect. This may be important for mechanical ventilation of patients, as injured or infected lungs may therefore be more vulnerable to mechanically induced effects on macrophages (Pugin et al. 1998). Upon stretch, macrophages also produce more NO, which can locally protect alveolar cells from stretch-induced apoptosis in vitro (Edwards et al. 2000). Surfactant in the lungs determines the surface tension on

cells in the alveoli, including macrophages. In knockout mice lacking surfactant protein B (SP-B), alveolar macrophages experienced increased tension, which resulted in flattening of the cells and a decrease in bead phagocytosis. Repletion with SP-B restored phagocytosis levels (Akei et al. 2006). Wu and colleagues sought to find the mechanism behind stretch-induced lung inflammation and found in mouse alveolar macrophages that reactive oxygen species (ROS) production was increased upon ventilation. ROS was shown to activate the NLRP3 inflammasome, which in turn activated caspase-1. Caspase-1 cleaves cytokines pro-IL1-β and pro-IL-18 to render them active (Wu et al. 2013). Increased pro-inflammatory cytokine release could then lead to local inflammation. Interestingly, this process was also dependent on TLR4 signaling, a pathway already mentioned above as mechanosensitive to substrate stiffness. TLR4 signaling may thus be a general mechanosensing pathway in macrophages.

Stretch was also studied in macrophage types that are not directly related to lung tissue. Strain on PMA-treated primary human monocytes and U937 macrophage-like cells led to increased production of specific matrix degrading enzymes, potentially initiating a cellular response to stretch to alter the local ECM (Yang et al. 2000; Matheson et al. 2006). In peritoneal macrophages, static strain of 20% increased expression of chemokines and cytokines; treatment with LPS even synergistically enhanced cyclooxygenase 2 (COX-2) and IL-6 expression (Wehner et al. 2010). In contrast, applying cyclic biaxial strain to BMDMs or RAW264.7 cells in combination with titanium particles did not affect pro-inflammatory genes (but it did in osteoblasts) (Lee et al. 2013). Oya et al. showed that oxygen levels can influence the response of macrophages to stretch: PMA-differentiated THP-1 macrophages, which were subjected to 10% strain at 1 Hz for 24 h, displayed stretch-induced elongation and orientation, but this effect was suppressed under hypoxic conditions, where hypoxia-inducible factor 1-α (HIF1-α) expression was increased (Oya et al. 2013).

In conclusion, next to static mechanical cues, macrophages can also be subjected to dynamic mechanical signals like stretch. Stretch adds another determining factor from the mechanical environment and may direct tissue-specific macrophage populations to a unique phenotype and function.

9.2.2.5 Macrophage Response to Implants

Since macrophages are involved in tissue remodeling, they also play an important role in the foreign body response to surgical implants. Just like monocytes, macrophage activation can be influenced by mechanical characteristics of the implant, and this has been studied using a variety of implantation-compatible materials. Among others, it has been shown that macrophages can respond to surface topography of titanium. It was shown that cell shape of J774A.1 mouse macrophages is affected by the roughness of titanium surfaces. Titanium surface roughness also increased cytokine and NO production and expression of bone morphogenetic protein 2 (BMP-2), a protein important in bone development, which is required

for proper incorporation of certain implants (Takebe et al. 2003; Tan et al. 2006). RAW264.7 macrophages also responded to titanium surface roughness or nanotube layer topography, among others, by altering BMP-2 expression (Refai et al. 2004; Sun et al. 2013). J774A.1 macrophages on nanostructured titanium were restricted in their movement and showed a reduction in cytokine and NO production (Lee et al. 2011). However, in these studies, different techniques were used for preparation of titanium topography, and the geometry of the surface topography may therefore differ. This might explain the discrepancy of the titanium topography-dependent effect on cytokine production found in these studies mentioned above. Looking deeper into macrophage activation, Hotchkiss and colleagues found that smooth titanium induced a more inflammatory M1-like phenotype in macrophages, whereas rough titanium induced an immunoregulatory M2 phenotype (Hotchkiss et al. 2016).

Besides titanium, many more materials with different topographies were used to study macrophage response. Primary human macrophages showed alterations in cytokine and gene expression on microstructured, but not nanostructured, polyvinylideenfluoride (PVDF) substrates (Paul et al. 2008). On silica substrates, rat peritoneal macrophages displayed higher phagocytosis on nanogrooves compared to flat surfaces (Wojciak-Stothard et al. 1996). Stainless steel and cobalt–chromium alloys elicited differences in cytokine production and giant body cell formation in RAW264.7 macrophages, depending on the surface topography (Anderson et al. 2016). Using poly-L-lactic acid (PLLA) fibrous surfaces, cytokine production by RAW264.7 macrophages was largely dependent on the diameter of the PLLA fiber on the surface (Saino et al. 2011). Tantalum-based nanodotted surfaces affected the morphology, focal adhesions, and cell spreading area of murine macrophages and macrophage-derived foam cells, as well as cytokine and chemokine production in these macrophages (Mohiuddin et al. 2012). Growing human primary macrophages on perfluoropolyether (PFPE) microstructures with multiple different topographies, Bartneck et al. showed that macrophages on regular grooves display an M1-like phenotype, whereas cells on larger cylindrical posts had an M2 phenotype. Very important for the macrophage response in this case was the periodicity of the structures (Bartneck et al. 2010). Wrinkled polyethylene surfaces led to induction of a more M2-like phenotype in murine BMDMs compared to flat surfaces (Wang et al. 2016).

Also substrate dimensionality is an important factor for macrophage activation. Bartneck and colleagues compared polylactic-co-glycolic acid (PLGA) fiber gels in a 2D and a 3D context. Whereas in a 2D setting, macrophages displayed a high production of pro-inflammatory cytokines, in 3D nanofiber networks, they displayed a high production of pro-angiogenic molecules (Bartneck et al. 2012).

9.2.2.6 Macrophage Response to Flow

Fluid flow has been shown to be another determinant in tissue macrophage function. For instance, in the context of the lung, mechanical flow has been shown to increase aggregation in HL-60 cells and primary human alveolar macrophages. However,

when artificial surfactant protein was added to the culture, aggregation was decreased in a concentration-dependent manner (Mita et al. 2001). Furthermore, it has been shown that macrophages are important for clearance of coagulation factor complexes in the bloodstream. Coagulation factor VIII and von Willebrand factor (VWF) form complexes in the bloodstream, and only under shear flow conditions macrophages are able to internalize this VIII–VWF complex (Castro-Nunez et al. 2012).

Macrophages are present in high numbers in atherosclerotic plaques, significantly contributing to disease pathology. Flow has been shown to modulate local macrophage phenotype. In a study where histology of murine atherosclerotic lesions subjected to different types of flow were studied, macrophages in the tissue with low shear stress had increased M1 inflammatory macrophage markers, whereas macrophages in oscillatory shear stress-subjected areas displayed a more M2-like phenotype (Seneviratne et al. 2015).

9.2.2.7 Mechanobiology in Macrophages: Comparison Between Different Studies

Studies investigating the effects of mechanical cues like substrate stiffness and stretch on macrophages in vitro often contradict each other with respect to the changes in macrophage phenotype and function. These differences may be explained by multiple factors. First, the magnitude or properties of the mechanical cue differ quite a lot between studies. For example, stiffness ranges studied vary from 0.3–200 to 100–800 kPa, and surface topographies include grooves, fibers, nanodots, pillars, or tubes. Furthermore, different substrate chemistries are used. For ECM stiffness studies, gels are made from polyacrylamide or PEG, materials that, for example, have different pore sizes and charges. For surface topography studies, effects are monitored on a wide variety of materials, like titanium, PLGA, PLLA, PVDF, and stainless steel. Yet, the most important determinant for the observed changes is most likely the fact that different macrophage cell types are studied. Human cell line THP-1 macrophages, murine BMDMs, RAW264.7 and U937 macrophage-like cell lines, and human monocyte-derived macrophages are all used in different studies. These cells are derived from distinct environments, meaning that, although they are all macrophages, they may be differently programmed and thus show variable responses to mechanical cues.

In conclusion, not only cell origin, tissue-specific biochemical signals, and state of the tissue (healthy, infected, or damaged) affect macrophages, but also mechanical signals have now clearly been demonstrated to determine macrophage phenotype. This makes the macrophage system complex, and a multidimensional model for macrophage development and classification seems therefore to be crucial for a complete understanding of macrophage biology (Okabe and Medzhitov 2016). Clearly, with the current evidence at hand, mechanical signals should be included in these types of models as key driving factors in macrophage phenotype.

9.2.3 Dendritic Cells

DCs, just like macrophages, are present in many peripheral tissues with different mechanical environments. However, whereas there is quite extensive literature on mechanobiology in macrophages, studies on effects of mechanical cues on DCs are far less numerous.

Studying the effect of stretch on DCs, Lewis and colleagues seeded immature murine DCs on different adhesive substrates and exposed the cells to stretch (Lewis et al. 2013). They found that stretch over a long period of time (12 h) significantly affected cell viability. Over short time periods, 3% stretch increased maturation markers, but not cytokine production in DCs on all adhesive substrates. Also, irrespective of adhesive substrate, stretched DCs induced a higher percentage of T-cell proliferation compared to non-stretched cells. Stretch thus seems to induce a semi-mature phenotype, which may be relevant for DC function in tissue environments undergoing cyclic strain, like vasculature or lungs.

Kou et al. studied the response of DCs to surface properties in the context of clinical titanium implants. Human immature monocyte-derived DCs (moDCs) were cultured on titanium surfaces with different roughness and surface energy (Kou et al. 2011). They showed that DCs on smooth and rough surfaces both displayed a mature phenotype, but that surface chemistry together with roughness promoted an immature phenotype, which is beneficial for reducing inflammation upon implantation of this type of implants.

Van den Dries and colleagues seeded human immature moDCs on a variety of substrates, investigating the effect of substrate properties on formation of adhesion structures in DCs (van den Dries et al. 2012). They showed that formation of focal adhesions is influenced by physicochemical properties, but formation of protrusive actin-rich structures called podosomes is not. Interestingly, moDCs cultured on micropatterned substrates aligned their podosomes along the ridges of the patterns. In addition, DCs cultured on micropatterns seemed less responsive to pro-inflammatory lipid prostaglandin E_2 (PGE_2) as PGE_2-induced podosome dissolution observed on flat surfaces did not occur in DCs on the micropatterns. Also, they found that immature DCs on micropatterns showed higher internalization of bacteria. Substrate geometry thus not only affects maturation of DCs but also their specific functions in an undifferentiated state.

Just like in macrophages, not only the extracellular mechanical environment affects DC phagocytosis, also the mechanical properties of the target seem to play a role. Mathaes et al. showed in a DC cell line that geometry and size of antigens affect the extent of DC activation, as spherical nanoparticles induced higher expression of maturation marker proteins CD83 and CD86 than elongated nanoparticles or microparticles (Mathaes et al. 2015).

The effect of mechanical extracellular pressure on DC maturation was investigated by Craig and colleagues (Craig et al. 2009). They found that increasing pressure with 40 mmHg for 12 h resulted in increased production of IL-12, IL-6, TNF-α, and interferon-γ (IFN-γ) and increased maturation marker expression in

human immature moDCs. These DCs induced $CD4^+$ T-cell proliferation more efficiently than immature DCs cultured under ambient pressure. This might have consequences for in vivo situations with increased extracellular pressure, like inflammation sites or tumor sites, where immature DCs may be driven toward a mature phenotype. Whether pressure-treated immature DCs are still efficient in antigen recognition and uptake was not investigated. It may therefore be that driving these DCs toward a more mature phenotype by increased extracellular pressure may prevent them from efficient antigen recognition and in this way inhibiting generation of an adaptive immune response. In addition, Craig and colleagues showed that LPS-matured moDCs were more responsive to changes in pressure than cytokine cocktail-matured moDCs, since LPS-treated cells showed an increase in $CD8^+$ T-cell proliferation and IL-12 and TNF-α production (Craig et al. 2009). As such, in vitro generation of mature antigen-loaded human DCs that are used in DC immunotherapies may benefit from incorporating specific mechanical signals during culture.

Since DCs migrate from their origin in the bone marrow to peripheral tissues through the blood circulation and from peripheral tissues toward lymph nodes through the lymph vessels, DCs are also subjected to flow in distinct tissue compartments. The maturation status of DCs appears to be important for adhesion to the vessel wall under shear flow as immature human moDCs were found to adhere more efficiently to human umbilical vein ECs than mature DCs, regardless of the level of fluid shear stress (Jiang et al. 2005). This may be specific for blood vessels, since the ECs used in this study were blood vessel lining ECs. Whether mature DCs bind more efficiently to lymph vessel ECs has not been studied yet. In a microfluidic lymph node model, Moura Rosa and colleagues investigated the dynamics in DC–T-cell interactions in the lymph node under shear flow. They applied $CD4^+$ T cells or $CD8^+$ T cells in flow over a monolayer of a murine DC cell line and could show that antigen-specific T cells selectively bound to activated DCs only and that flow affects this cell–cell interaction. Increasing the flow up to 1.2 dyn/cm^2 for $CD8^+$ T cells or 12 dyn/cm^2 for $CD4^+$ T cells initiated detachment (Moura Rosa et al. 2016).

Besides being affected by flow in the lymph node, DCs themselves also influence the mechanical environment of the lymph node. Using mouse models, Acton and colleagues showed that C-type lectin-like receptor 2 (CLEC-2) protein on the membrane of the DCs binds to its ligand podoplanin (PDPN) on fibroblastic reticular cells (FRCs) in the lymph node, leading to stretch in FRC network. This stretch allows for lymph node expansion and in this way provides room for influx and proliferation of T cells, important for an efficient adaptive immune response (Acton et al. 2014).

9.3 Concluding Remarks

In conclusion, it has now been shown that mechanical cues from the extracellular environment, like ECM stiffness, ECM topography, and fluid flow, but also signals such as stretch or compression on the tissue scale can change cellular shape and affect

the phenotype and function of a variety of cell types. Unfortunately, most studies are still performed in 2D cell culture systems, and although in these systems, mechanical characteristics of substrates are easily manipulated, it does not completely reflect the complexity of real tissues. Future studies should therefore be directed at studying cellular functions in a 3D context. These studies will provide essential insight into the role of mechanical cues in a more physiological context of a tissue.

Macrophages, monocytes, and DCs are related immune cells that are present throughout the body and receive input through multiple mechanical signals. For macrophages, the effects of ECM stiffness, topography, stretch, and flow have been strongly established by multiple studies. Discrepancies in results among studies may be explained by the use of macrophages of different origins. The few studies on mechanical effects in DCs indicate that, like macrophages, DCs are also mechanosensitive. Shear stress from blood flow is a crucial factor in monocyte functioning, regulating monocyte adhesion to ECs and subsequent transendothelial migration. Monocytes are a very plastic cell type and can be used to generate human macrophages or DCs in vitro. However, although these monocyte-derived macrophage and DC systems are widely used models, it remains to be determined whether this monocyte plasticity could also be manipulated by changing the mechanical environment during differentiation.

Although it has been long established that differences in mechanical signals can lead to changes in cell phenotypes, the intracellular pathways involved in translating these mechanical cues into a cellular response are still largely unknown, and new pathways are only scarcely being identified. A better understanding of these intracellular mechanisms of mechanotransduction can provide us with leads for molecular, also macrophage-based, therapies targeting diseased tissues with altered mechanical properties.

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Part III
Role of Macrophages in Disease

Chapter 10
Macrophages' Role in Tissue Disease and Regeneration

Lewis Gaffney, Paul Warren, Emily A. Wrona, Matthew B. Fisher, and Donald O. Freytes

Abstract Inflammation is an essential component of the normal mammalian host tissue response and plays an important role during cardiovascular and musculoskeletal diseases. Given the important role of inflammation on the host tissue response after injury, understanding this process represents essential aspects of biomedical research, tissue engineering, and regenerative medicine. Macrophages are central players during the inflammatory response with an extensive role during wound healing. These cells exhibit a spectrum of activation states that span from pro-inflammatory to pro-healing phenotypes. The phenotype of the macrophages can have profound influences on the progression of disease or injury. As such, understanding and subsequent modulation of macrophage phenotype represents an exciting target area for regenerative medicine therapies. In this chapter, we describe the role of macrophages in specific cases of injury and disease. After myocardial

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infarction, a biphasic response of pro- and anti-inflammatory macrophages are involved in the remodeling process. In volumetric muscle loss, there is an intricate communication between inflammatory cells and progenitor cells affecting repair processes. Osteoarthritis is characterized by increased levels of pro-inflammatory macrophages over an extended period of time with significant impact on the progression of the disease. By harnessing the complex role of macrophages, enhanced therapeutic treatments can be developed that enhance the normal healing response as well as help the survival of therapeutic cells delivered to the site of injury.

10.1 Introduction

Inflammation is an essential component of the normal mammalian host tissue response and plays a key role in the natural healing process following injury and a broad role in abnormal disease processes that lead to inferior healing. In particular, inflammation plays an important role during cardiovascular and musculoskeletal diseases with significant impact on human health. Cardiovascular diseases remain one of the major causes of death within industrialized nations, currently accounting for approximately 20 million deaths per year (Mozaffarian et al. 2016). Trauma to the extremities, which commonly includes volumetric muscle loss, frequently occurs in vehicle accidents causing long-term disability (Vos et al. 2012). Volumetric muscle loss injuries represent more than half of military injuries (Owens et al. 2007, 2008). In terms of other musculoskeletal tissues, more than 20 million people are afflicted by osteoarthritis in the United States alone (Felson 2004), while hundreds of thousands of cases of tendinopathies and ligament and tendon injuries occur each year (AAOS 2008; Beaty 1999; Cole et al. 2005). Importantly, since inflammation is a key component of the host tissue response after injury, expanding our understanding of this process, and of important cellular players, represents one of the most important aspects of biomedical research, tissue engineering, and regenerative medicine.

Macrophages are central players during the inflammatory response in all of these disease and injury processes, directing T-cell activation, promoting stem cell and progenitor cell migration, activating angiogenic responses, and guiding extracellular matrix remodeling. Given their extensive role during inflammation and wound healing, macrophages are capable of exhibiting a spectrum of activation states spanning from pro-inflammatory to pro-healing phenotypes that reflect the milieu of signals found at the site of injury or disease. This role is accomplished primarily through phenotype plasticity, allowing the same macrophage to switch between pro-inflammatory and pro-healing states depending on the surrounding environmental cues. The phenotype of the macrophages can have profound influence on the progression of disease or injury. Figure 10.1 shows a very simplistic representation of the progression of macrophage activation in tissue repair and during disease. As

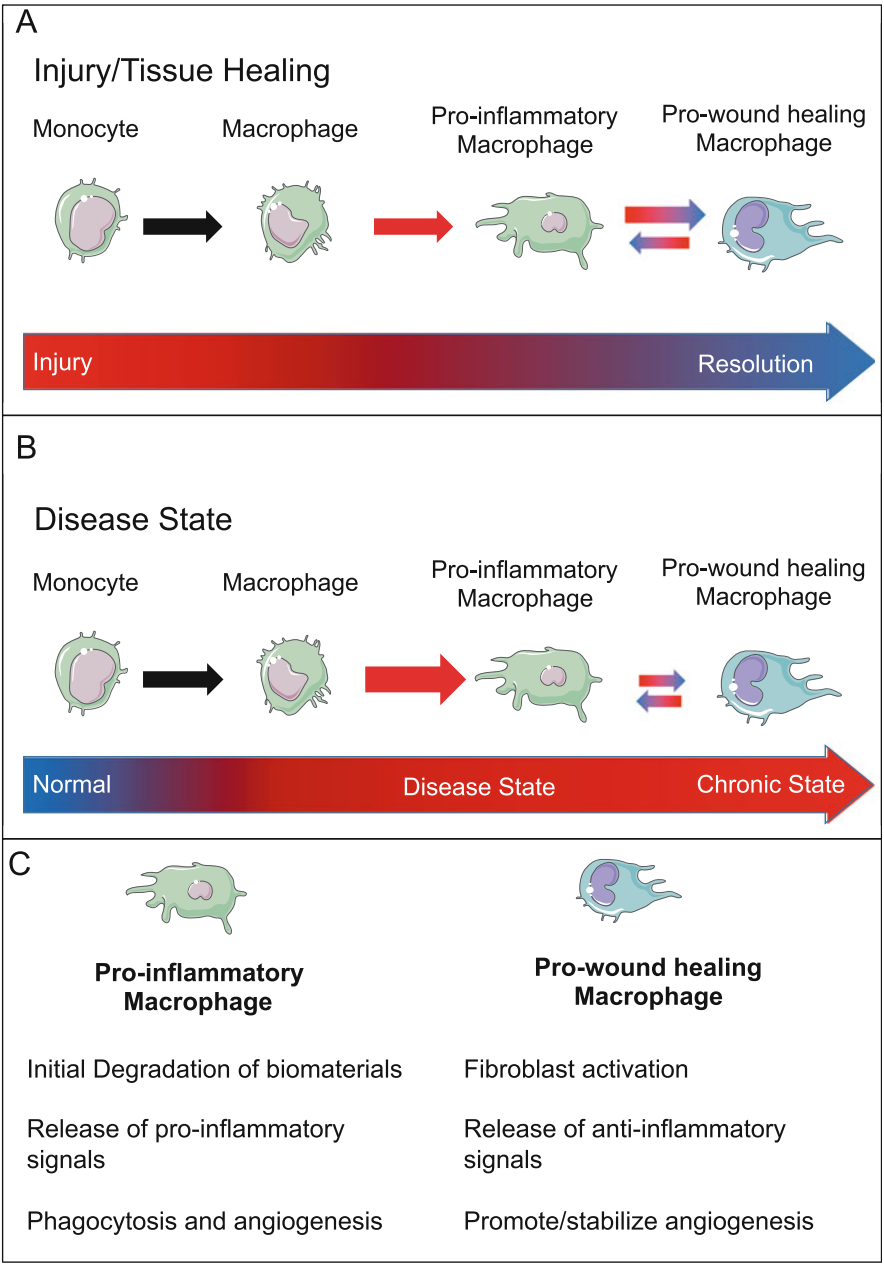

Fig. 10.1 Macrophage polarization during injury and disease. (**a**) During tissue injury and healing, there is an infiltration of circulating monocytes to the site of injury that subsequently differentiate into macrophages. Throughout the progression of the healing response, macrophages become activated and show a pro-inflammatory phenotype (M1-like). As the response continues, there is a typical shift toward a pro-wound healing phenotype (M2-like) often leading to fibrosis or the resolution of the inflammatory response. (**b**) During the disease state, this process is altered with often a significant persistence of a pro-inflammatory response leading to disease or a chronic

such, understanding and subsequent modulation of macrophage phenotype represents an exciting target area for regenerative medicine therapies.

In this chapter, we will explore the role of macrophages in specific cases of injury and disease. First, we will provide a brief overview of macrophage polarization within the context of injury and disease. Next, we will describe the participation of macrophages in heart disease. Then, we will turn our attention to skeletal muscle disease and volumetric muscle loss. Finally, we will examine musculoskeletal soft tissue diseases, such as arthritis, and traumatic injuries. The chapter will conclude by acknowledging current gaps in scientific knowledge and suggesting areas requiring increased focus in the future.

10.2 Macrophage Polarization

The immune system is a complex and dynamic environment composed of several cell types, cytokines, and their interactions, which involve diverse biochemical pathways. An integral part of the immune system is the macrophage, a large, phagocytic cell often part of the inflammatory and reparative responses to injury. Originating from monocytes produced in the bone marrow, blood-derived macrophages, along with tissue-resident macrophages, have roles in a variety of cell processes such as homeostasis, inflammation in response to disease or injury, and reparative processes such as tissue repair, chemotaxis, angiogenesis, and clearance of cellular debris and necrotic cells (Wynn et al. 2013).

When injury occurs, neutrophils are the first immune cells recruited to the site, where they begin to clear out cellular debris via phagocytosis and the release of proteases (Wright et al. 2010). As they undergo apoptosis and clear out cellular debris, neutrophils begin to release signals that recruit circulating monocytes and tissue-resident macrophages. Monocytes are of the hematopoietic lineage, originate from myeloid progenitors in the bone marrow, and can be recognized in human peripheral blood by the positive expression of CD14 (Passlick et al. 1989). These cells circulate throughout the blood as well as reside in tissues as reserve cells that can be easily mobilized when injury occurs (Nahrendorf and Swirski 2013). In fact, in a mouse model of myocardial infarction, it has been estimated that about 50% of the monocytes recruited to the site of injury originate from the spleen, making them one of the most abundant cell types in the early stages of inflammation in infarcted tissue (Swirski et al. 2009).

Fig. 10.1 (continued) inflammatory state. (**c**) For simplicity, macrophage phenotypes can be categorized into two major subtypes typically associated with pro-inflammation and pro-wound healing. These two subcategories can also be found using the previous nomenclature such as M1 (classically activated) and M2 (alternatively activated). Although this is an oversimplified view of the spectrum of activation possible with macrophages, for the purpose of this chapter, we will refer these to broad categories as M1-like and M2-like. Art adapted from Servier Medical Arts

Monocytes are differentiated to macrophages once recruited to the site of injury or when in contact with pathogens or activating signals (Lambert et al. 2008; Mantovani et al. 2002). Studies have also shown that monocyte colony-stimulating factor (M-CSF) (Hamilton 2008) and vascular endothelial growth factor (VEGF) are strong recruiters of monocytes (Lambert et al. 2008). Adhesion molecules on the cell surface allow monocytes to adhere, roll, and migrate to sites of injury across the surface of blood vessels where they begin the differentiation process toward macrophage cells (Shi and Pamer 2011; Ley et al. 2007). Although macrophages demonstrate a large amount of heterogeneity within currently defined activation states (Eligini et al. 2015; Lawrence and Natoli 2011), they can be generally classified into two broad categories: classically activated (M1-like or pro-inflammatory) and alternatively activated macrophages (M2-like or pro-healing). Given the numerous activation states found in vivo, it is difficult to assign a specific name or classification since their response is tied to the types of signals used to activate them (Murray et al. 2014). For simplicity, we will use the generic M1-like and M2-like terms to describe the broader classification. During injury, these two types of macrophages arrive at the site in a biphasic manner with M1-like macrophages appearing first. M1-like macrophages are typically activated by interferon-γ (IFN-γ) and can be polarized in vitro by the addition of IFN-γ and lipopolysaccharide (LPS). These polarized macrophages release inducible cytokines typically associated with inflammation such as IL-1β and TNF-α (Brown et al. 2012). However, there are species differences that need to be taken into account such as the significantly higher expression of inducible nitric oxide synthase (iNOS) in mouse macrophages when compared to human (Spiller et al. 2016). These activities promote further recruitment of immune cells to the site of injury, provide initial resistance against pathogens, and provoke a pro-inflammatory response dependent on the tissue where they reside.

Following the pro-inflammatory macrophage response, a gradual shift toward more pro-healing macrophage phenotype occurs (Frangogiannis 2014; Mosser and Edwards 2008). There are also several subsets of M2-like macrophages that have been described (Mantovani et al. 2002, 2004) such as M2a, M2b, and M2c. M2a macrophages can be polarized in vitro by interleukin-4 (IL-4) and interleukin-13 (IL-13), while M2b macrophages are polarized by IL-1R (Gordon 2003). M2c macrophages are activated by IL-10 (Mantovani et al. 2004). However, as mentioned above, this nomenclature has been shown to be over simplistic, and a new naming convention is being explored (Murray et al. 2014). M2-like macrophages are typically associated with tissue repair given their angiogenic activities, fibrotic responses, and stimulation of production of extracellular matrix (ECM) components. However, this M1 to M2 biphasic recruitment pattern is highly simplified, and there are exceptions to using this as characterization for an inflammation response, such as tissue remodeling in muscular dystrophy (Villalta et al. 2009), insulin resistance (Olefsky and Glass 2010), and advanced age (Mahbub et al. 2012). Recruited monocytes fail to differentiate adequately in these conditions which results in a combination of macrophages with variable polarization states, therefore leading to unsuccessful remodeling and tissue repair (Carvalho et al.

2013; Villalta et al. 2011a, b). It has been suggested that promoting a balance of macrophage subtypes could improve the outcomes of these conditions (Carvalho et al. 2013; Villalta et al. 2011a, b) highlighting the importance of macrophage polarization in disease states.

10.3 Cardiac System

10.3.1 Macrophages in Cardiac Muscle Diseases

As deterioration and irreversible damage can occur rapidly in cardiovascular diseases (CVD), quick and early detection is required in order to minimize damage. Examining the immune environment, including the composition and activation of the macrophages present at the site of injury, may provide clues related to the progression of disease as well as important information for the repair of the damaged tissue due to their relatively early response in the inflammatory process. There are several forms of CVD including congenital, viral, and self-inflicted disease states. In this chapter, we will only touch on a few of these conditions that occur more frequently throughout industrialized nations: myocardial infarction, myocarditis, and the broad category of cardiomyopathies. Macrophages play an important role in both the inflammatory response and reparative processes in these conditions.

One of the leading causes of death in the United States and other developed countries is the occurrence of a myocardial infarction (MI) or heart attack (Mozaffarian et al. 2015). Over time, fat and plaque accumulate in the coronary arteries and eventually block the delivery of blood and oxygen to the heart tissue (Robbins et al. 2010). Although this process is also driven in part by macrophages (Moore et al. 2013), we will focus on the role of macrophages within the myocardial tissue itself given its importance for designing regenerative medicine therapies. As the myocardium becomes deprived of oxygen and nutrients, there is decreased production of adenosine triphosphates (ATP) that supply energy, leading to an increase in necrotic tissue and a cellular shift toward inflammation and fibrosis (Frangogiannis 2008). This fibrotic response leads to substantial myocardial remodeling that diminishes heart function, may cause arrhythmias, and can ultimately lead to heart failure (van Amerongen et al. 2007; Troidl et al. 2009). These processes are summarized in Fig. 10.2.

Although essential, macrophages may themselves cause additional damage. They can further contribute to the accumulation and stability of atherosclerotic plaques by providing enzymatic activity that favors plaque formation (Belcastro et al. 2015; Stoger et al. 2012; Medbury et al. 2014). Reperfusion therapy, the restoration of oxygenated blood to ischemic tissue, may also accentuate the inflammatory response. During reperfusion of the tissue, immune cells are recruited that are capable of contributing to further injury by releasing enzymes and reactive

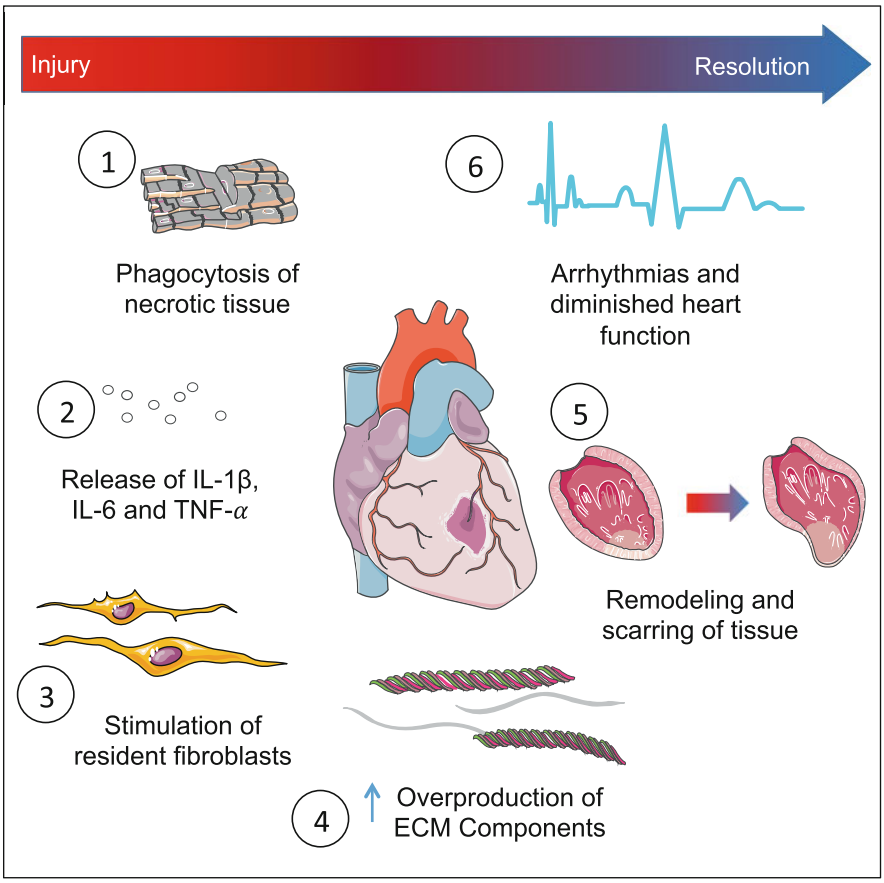

Fig. 10.2 The inflammation response after myocardial infarction. This schematic represents some of the important events in the macrophage-mediated inflammatory response after myocardial infarction. Monocytes are recruited to the site of injury from resident tissues and peripheral blood as a result of signals released from necrotic cells (1) and then differentiate into macrophages and further polarize into pro-inflammatory and pro-healing macrophages. There's an initial release of inflammatory cytokines (2) that initiates the myocardial remodeling. After the initial inflammatory response, there is a shift toward a pro-healing macrophage response that leads to the activation (3) of fibroblast and the remodeling of the extracellular matrix (4). Changes in the overproduction of collagen leads to the formation of scar tissue and the remodeling of the myocardial wall potentially leading toward heart failure (5 and 6). Art adapted from Servier Medical Arts

oxygen species (ROS) that degrade tissue and can induce cell death, autophagy, and necrosis (Kempf et al. 2012; Yan et al. 2013; Eltzschig and Eckle 2011). There is currently an interest in further exploring reperfusion therapy by attenuating the potential inflammatory effects with the use of pentraxin 3 (PTX3) produced from bone marrow-derived cells (Shimizu et al. 2015), secreted frizzled-related protein 5 (Sfrp5) (Nakamura et al. 2016), or macrophage migration inhibitory factor (MIF)

(Pohl et al. 2016; Stoppe et al. 2015). Reperfusion therapy is a time-dependent process, and further understanding is necessary to reintroduce it as a successful treatment.

Myocarditis is another form of CVD where early diagnosis of the condition can help prevent irreversible damage. It is described as the inflammation of the myocardium and is most commonly caused by a viral infection, but may also be a result of toxic agents or an autoimmune condition (Sagar et al. 2012; Biesbroek et al. 2015). In the process of clearing infected myocardium, macrophages are activated by signals such as TGF-β1 (Gong et al. 2012), which contribute to post-infarction fibrosis by promoting the expression of extracellular matrix (ECM) genes and suppressing expression of matrix metalloproteinases (MMPs) (Nakamura et al. 2000; Leask and Abraham 2004; Amoah et al. 2015). It has also been found that silencing microRNA-155 affects polarization of the macrophages present at the site of viral myocarditis infection by increasing levels of M2-like macrophages and decreasing levels of M1-like macrophages, suggesting a possible target for treatment (Zhang et al. 2016).

Other cardiomyopathies, such as diabetic cardiomyopathy and stress (or Takotsubo) cardiomyopathy, show similar patterns of macrophage behavior, and there are further studies aimed at understanding and controlling these inflammatory environments. For instance, Guo and colleagues investigated the effect of triptolide (a diterpenoid epoxide) from the *Tripterygium* root on a diabetic cardiomyopathy mouse model and found it suppressed the release of the inflammatory cytokines TNF-α and IL-1β, suggesting a protective effect by decreasing collagen deposition and attenuating fibrosis (Guo et al. 2016).

10.3.2 Tissue Repair of Cardiac Muscle

Cardiac tissue has a unique ECM structure and architecture that is very pliable and allows for the constant contraction of cardiomyocytes while generating the necessary force to circulate blood. The components of cardiac ECM include collagens, glycoproteins, glycosaminoglycans (GAGs), and proteoglycans (Rienks et al. 2014). As ischemia sets in from the deprivation of oxygen and other nutrients to the myocardium, a tissue remodeling response occurs by altering the ECM composition, primarily led by the M2-like macrophages present after the initial inflammatory response. While the heart does have a minimal regenerative capacity that may aid in a beneficial way, most often the result is a fibrotic scar, which is an example of harmful remodeling of damaged myocardial tissue. Macrophages release factors, such as TGF-β1 (Roberts et al. 1986) and PDGF (Shimokado et al. 1985), which activate resident fibroblasts to begin their differentiation into myofibroblasts (Murray and Wynn 2011). The fibrotic responses are, in part, caused by an excessive production of collagen I by myofibroblasts within the ECM, resulting in a collagen-rich scar at the infarction region (de Haas et al. 2014). This progressive scar eventually decreases cardiac output and may lead to heart failure.

Given the diminished capacity of the heart muscle to repair itself after an MI and fibrotic scar, tissue engineering can provide a therapeutic alternative that can replace the muscle loss and restore function. One approach is to engineer a cardiac patch using a biomaterial component to help mechanically stabilize the patch and a cellular component to help the reparative process (Domenech et al. 2016). There are several examples of this approach. Duan and colleagues used decellularized heart ECM and collagen to make hydrogels that could induce the differentiation of human embryonic stem cells into cardiomyocytes (Duan et al. 2011). The naturally derived gel was able to aid in the cardiomyocyte differentiation without addition of growth factors or cytokines. Similarly, Zhang and colleagues described a tissue-engineered cardiac patch with human embryonic stem cell-derived cardiomyocytes, fibrinogen, and Matrigel (Zhang et al. 2013). Characterization of these patches included electrophysiological properties, cardiac gene expression, ability to conduct action potentials, and contractile properties. Both studies show examples of the use of human cardiac cells with a naturally occurring biomaterial to create a functional cardiac tissue unit. Miyagawa and colleagues designed cardiac sheets comprised of poly(N-isopropylacrylamide)-grafted polystyrene and neonatal rat cardiomyocytes (Miyagawa et al. 2005). After two weeks postimplantation into a rat model, there was evidence of engraftment, angiogenesis, and improvement in cardiac function. Using a different approach, Jang and colleagues used 3D printing and a stem cell bioink to create a patch that promoted angiogenesis, reduced fibrosis, and increased cardiac function (Jang et al. 2016). There are several more examples of tissue-engineered constructs being designed and tested; however, regardless of the method used, any cellular and tissue component will inevitably interact with the inflammatory response found at the site of MI prior to implantation. In other words, any cardiac tissue-engineered construct will interact with polarized macrophages with potential impact on their therapeutic potential.

In order to ensure success of any cardiac tissue engineering approach, it is important to understand how these tissue-engineered constructs will interact with macrophages present at the site of injury (Freytes et al. 2012). There are currently few studies that directly look at the effect of macrophages on the behavior, gene expression, and protein production of tissue-engineered constructs. Many studies simply look for cases of necrosis and fibrosis or test for biocompatibility. Understanding the macrophage-engineered construct interactions may help optimize current patch designs and help predict their survival within the inflammatory environment caused by the disease and the surgical intervention. For example, mesenchymal stem cells (MSCs) have been widely explored as a cellular therapy for MI. MSCs injected into animal infarct models have been shown to increase M2-like macrophage levels via the release of IL-10 and decrease M1-like macrophage levels by decreased expression of IL-1β and IL-6 (Dayan et al. 2011; Cho et al. 2014). Additionally, macrophages conditioned with cardiosphere-derived cells (isolated from cardiac biopsy specimens) and added to a site of myocardial infarction within 20 min of reperfusion were shown to alter the inflammatory environment by reducing the infarct size while, interestingly enough, expressing

markers that do not strictly identify with either the M1 or M2 subtypes (de Couto et al. 2015). In vitro studies have also shown how M1-like macrophages could have detrimental effects on the survival of MSCs (Freytes et al. 2013) and how MSCs can regulate macrophage activation (Patricia et al. 2017).

As stated previously, cardiomyocytes derived from human pluripotent stem cells (hPSCs) can also provide another source of therapeutic cells to repair damaged myocardial tissue (Domenech et al. 2016). Pallotta and colleagues studied the interactions between macrophages and human embryonic stem cell-derived cardiomyocytes (hESC-CMs) and showed a BMP-mediated recruitment of M1 macrophage (Pallotta et al. 2015). The same study detected low levels of BMP being secreted by polarized macrophages with potential to affect progenitor populations that have not fully differentiated toward mature cardiomyocytes. How these interactions will affect an engineered cardiac patch remains to be determined but highlights the importance of macrophage interactions with engineered constructs. The response of macrophages to the site of injury is a heterogeneous and complicated process that needs to be better understood in order to design future tissue-engineered therapies for heart repair.

10.4 Skeletal Muscle System

10.4.1 Macrophage Response to Normal Loading

In response to normal loading, muscle tissue is under a constant state of damage and remodeling. Normal remodeling in muscle is very dependent on the timing of the response of M1-like macrophages and M2-like macrophages. In skeletal muscle, pro-inflammatory macrophages recruit satellite cells (resident progenitor cells) and remove damaged muscle cells via phagocytosis. Pro-wound healing macrophages then encourage the differentiation of the satellite cells into mature muscle cells (Brown et al. 2012; Saclier et al. 2013).

In muscle, M1-like macrophages secrete cytokines such as TNF-α, IL-1β, and IL-6, which are chemoattractants for muscle progenitor cells (Brown et al. 2012). This results in an accumulation of progenitor cells at the site of the injury. However, these chemokines also inhibit differentiation of the progenitor cells, and the reactive oxygen species that come along with the inflammatory response can be cytotoxic (Brown et al. 2012). Following an acute injury, early elimination of the macrophage response via toxins negatively impacts tissue regeneration. However, when the response is eliminated later on in the wound healing process, tissue regeneration is not impacted as much (Wang et al. 2014).

The transition to M2-like macrophages is critical to muscle regeneration (Brown et al. 2012). The transition ends the production of inflammatory reactive oxygen species (ROS). IL-10 is directly implicated in the transition from M1-like cells to M2-like cells and also drives the fusion of myoblast cells into myotubes (Brown

et al. 2012). IGF-1 (insulin-like growth factor) is another growth factor that shifts the immune response from pro-inflammatory to pro-healing. Tonkin and colleagues found that in mice in which IGF-1 was inhibited, macrophages were present but were mostly M1-like; this was accompanied by impaired fiber regeneration characterized by smaller fiber diameters, more fat deposits, and greater interstitial space after 10 days (Tonkin et al. 2015). Other proteins, such as AMPKα1, act as crucial intermediates for the transition from M1-like to M2-like phenotypes (Mounier et al. 2013). To show the importance of the shift to M2-like macrophages, Ruffel and colleagues blocked M2-associated genes (IL-10, Msr, IL13ra, and Arg-1), but not M1 genes (IL-1, IL-6, TNF-α, and IL-12), by inhibiting C/EBPβ, a regulator protein which induces upregulation of M2-like genes. At the site of injury, there was adequate clearance of cells and recruitment of progenitors; however, without the M2-like shift, the tissue was highly fibrotic (Ruffell et al. 2009). Collectively, these studies provide examples of impaired tissue regeneration without a transition from M1-like to M2-like macrophages.

10.4.2 Macrophages in Skeletal Muscle Disease

Diseased states of skeletal muscle have altered macrophage responses, which often result in amplification of the symptoms. Skeletal muscles affected by dystrophies, obesity, and aging have an abnormal inflammatory responses, which can lead to limited tissue regeneration and, in cases of dystrophies, degeneration of the native tissue.

10.4.2.1 Muscular Dystrophies

In muscular dystrophies, the shift from M1-like to M2-like macrophages is delayed, often resulting in degeneration of muscle (Zordan et al. 2013). With a prolonged exposure to pro-inflammatory macrophages, muscles undergo degeneration due to the excessive removal of tissue rather than the healthy response brought about by activation of M2-like macrophages (shown in Fig. 10.3).

Understanding the prolonged M1-like stage in dystrophic muscles can lead to targeted therapies that could offset the symptoms of the disease. Capote and colleagues showed that ablation of osteopontin (OPN), an integrin-binding protein highly expressed in dystrophic muscles, helps the M1-like to M2-like transition in dystrophic models (Capote et al. 2016). Mojumdar and colleagues showed that inhibiting CC chemokine receptor 2 (CCR2), which binds monocyte chemoattractant proteins and is upregulated in cases of muscular dystrophy, decreased the amount of macrophage cells recruited to the muscle (Mojumdar et al. 2014). The same group also looked at the effect of toll-like receptor 2 (TLR-2) in mouse models with Duchenne muscular dystrophy (DMD). Interestingly, inhibition of TLR-2 reduced the infiltration of macrophages and led to a

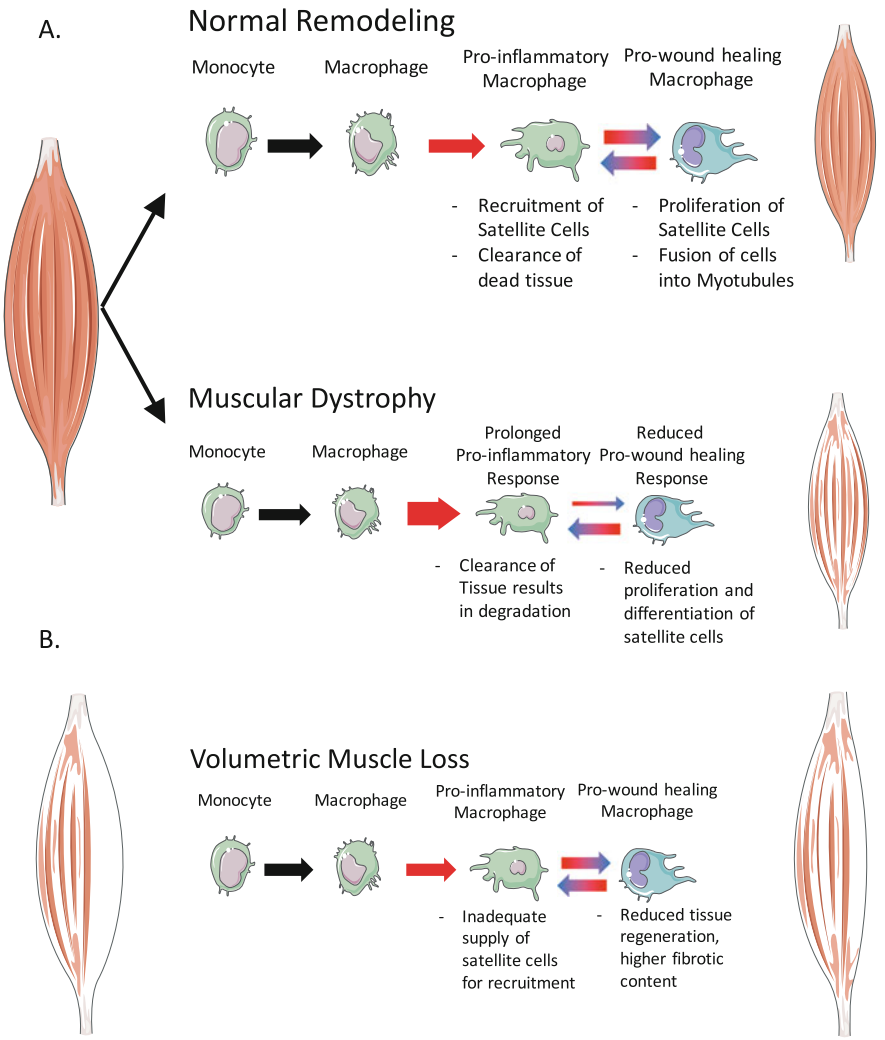

Fig. 10.3 (**a**) Normal response to loading results in fully repaired muscle tissue; however, in cases of muscular dystrophies, tissue degenerates and becomes highly fibrotic due to an excessive M1-like cell population. The M1-like macrophages continually clear out tissue, and because of a decreased population of M2-like cells, the progenitor cells are not differentiated. (**b**) In the event of volumetric muscle loss injuries, a lack of cells in the injury area inhibits full regeneration due to low satellite cell recruitment; the tissue has a decreased volume and an increased fibrotic content. This results in decreased muscle function. Art adapted from Servier Medical Arts

decreased regenerative response in normal animals. However, in dystrophic mice, the inhibition of TLR-2 was found to enhance the wound healing response, which was characterized by less necrotic injury, decreased fibrosis, and larger myofibers (Mojumdar et al. 2016). Although not fully understood, the effects of macrophage

plasticity and its implication in muscular dystrophies could provide insights into possible avenues for treatment/management.

10.4.2.2 Obesity

In obese individuals, skeletal muscle has an altered immune response when compared to muscle in individuals with a healthy weight. This alteration can have implications in terms of insulin resistance and remodeling in adipose tissue. In muscle cells of mice on a high-fat diet (which increased levels of myostatin), the muscle cells expressed inflammatory cytokines and increased insulin resistance compared to mice on a high-fat diet with myostatin inhibited (Dong et al. 2016). Accumulation of M2-like macrophages was also shown to increase the insulin sensitivity (Ikeda et al. 2013). In terms of macrophage phenotype in human patients, pro-inflammatory gene ITGAX (CD11c) correlated positively with fasting blood glucose, while anti-inflammatory markers TGF-β1 and CD163 were negatively correlated with fasting blood glucose (Fink et al. 2013). These findings are also supported by in vitro studies of muscle cells challenged with fatty acids (palmitate), leading to increased expression of inflammatory pathways (higher expression of TNF-α, IL-6, and CCL2). This evidence suggests that fatty acids cause muscles to express pro-inflammatory regulators, which could then affect insulin resistance of the muscle cells (Pillon et al. 2012). Inflammation from macrophage cells may have altered effects in cases of obesity and play a role in insulin resistance.

10.4.2.3 Aging

Aging is also a risk factor for muscle degeneration and fibrotic infiltration. Similar to muscular dystrophies, muscle loss due to aging is likely caused by a prolonged inflammatory response, characterized by a higher expression of IL-1β and IL-Ra at rest. After exercise bouts, elderly subjects had a decreased macrophage response compared to younger subjects, indicating that they may have an impaired macrophage response to exercise as well (Przybyla et al. 2006). Aging causes muscle cells to express a pro-inflammatory response, which leads to more degeneration and fibrosis (Fink et al. 2013).

10.4.3 Macrophages in Skeletal Muscle Remodeling

In response to traumatic volumetric muscle loss (VML), the inflammatory and remodeling processes are even more important. One of the main challenges of volumetric muscle loss injuries is the lack of progenitor cells locally available for repair, which often leads to decreased volume after healing (Fig. 10.3). Due to the larger tissue space that needs to be filled after VML, M1-like macrophages need to

recruit satellite cells and clear damaged cells to allow for restoration of muscle. To examine the effect of scaffolds in an area that did not have an adequate supply of myogenic cells, Garg and colleagues compared the regenerative response in mice models treated with devitalized scaffolds via repeated freeze-thaw cycles or minced muscle grafts. The devitalized scaffold did not produce as many fibers when compared to vital muscle grafts at both the early and late time points. An in vitro comparison of macrophage response showed that the vital grafts initially supported a mix of M1-like and M2-like macrophages, but at the end of 4 days of culture, the macrophage population was almost solely M2-like macrophages. In devitalized scaffolds, macrophages were not differentiated to M2-like cells. Interestingly, in functional testing in vivo, the devitalized scaffolds still led to a higher isometric torque in the repaired muscle than in the untreated group, suggesting a more complex series of in vivo events. The authors concluded that the distance from the cell source should be considered when using devitalized scaffolds. As long as there is a source of myogenic cells, the scaffolds could improve the regenerative response (Garg et al. 2014).

However, if the M1 response lasts too long, the new tissue is highly fibrotic, leading to decreased function. Aspirin is a COX 1/2 inhibitor, which has been shown to impede the regenerative response by affecting the transition to M2-like macrophages (Dearth et al. 2016). In in vitro culture of THP1 cells (a human monocytic cell line), treatment with aspirin caused a 91% reduction in expression of CD206 (pro-healing marker), which was coupled with an increase of CD86 (pro-inflammatory marker). In rat studies of abdominal muscle defects, both collagen deposition and myogenesis were greatly reduced after 35 days post injury in animals that were treated with aspirin. These results challenge the efficacy of aspirin in injuries that require an intense remodeling response in vivo and link the importance of the phenotypic switch to myogenesis and collagen deposition (Dearth et al. 2016). Another concern with VML injuries is the lack of required new vasculature. In most normal muscle remodeling, vasculature is not damaged. However, in VML, there is damage to the vasculature. In mice with decreased number of macrophages (e.g., ablated via clodronate), endothelial cells underwent an endothelial to mesenchymal transition, which could explain the decrease in angiogenesis observed, leading to more cells producing collagen and a higher fibrotic content (Zordan et al. 2014). Cell recruitment, transition to M2-like macrophages, and formation of new vasculature are all needed in cases of VML and should be considered for treatment of these injuries.

10.5 Musculoskeletal Soft Tissue

10.5.1 Macrophages in Musculoskeletal Soft Tissue Disease

Macrophages can also contribute to musculoskeletal soft tissue disease progression. Osteoarthritis (OA) is a pathological remodeling of tissues in the affected joint

induced by mechanical stress and pro-inflammatory cytokines. Typical features of joints with OA include degradation of articular cartilage, hypertrophy of subchondral bone, degeneration of ligaments, and inflammation of the synovium (Loeser et al. 2012), the connective tissue lining the inner surface of the joint capsule. The progression of OA is driven by the abnormal regulation of inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and extracellular matrix (ECM)-degrading enzymes including matrix metalloproteases (MMP) and ADAMTS (a disintegrin and metalloprotease with thrombospondin type 1 motifs) (Bondeson et al. 2010; Loeser et al. 2012; Kapoor et al. 2011). As a result, articular chondrocytes within the joint lose their characteristic phenotype, expressing molecules associated with hypertrophy and terminal differentiation (Lories and Luyten 2011). The subsequent changes in microstructure become increasingly severe. For example, osteoarthritic synovium can lead to reduced glycosaminoglycan (GAG) content in articular cartilage (Beekhuizen et al. 2011), exposing type II collagen normally shielded from degradation by GAG-rich proteoglycans to protease activity. The resulting collagen breakdown stimulates the production of inflammatory cytokines that further inhibit proteoglycan and type II collagen synthesis (Bondeson et al. 2010). Major osteoarthritic changes at the molecular, cellular, and tissue levels are depicted in Fig. 10.4.

Macrophages are primary sources of IL-1β and TNF-α, among other cytokines, making them key drivers of inflammatory progression in osteoarthritic joints. They originate from circulating monocytes that migrate to the source of the inflammatory signals via chemotaxis and ultimately become resident cells. Synovial tissue from patients with more advanced OA has shown higher numbers of resident macrophages compared to early-stage osteoarthritic synovial tissue, suggesting an elevated recruitment of cells (Benito et al. 2005). Over time, recruited macrophages also express other inflammatory signals and cause alterations in local microstructure. Over 21 days of co-culture with cartilage, synovial macrophages produced high levels of the pro-inflammatory cytokines IL-6 and IL-8 and osteoprotegerin, a receptor for the tumor necrosis factor family of ligands (Beekhuizen et al. 2011). Macrophages also secrete factors that act synergistically with pro-inflammatory molecules. Released in the presence of IL-1β, high mobility group box 1 (HMGB1) protein participates in the chemotactic recruitment of osteoblasts and osteocytes (Taniguchi et al. 2007), altering normal subchondral bone homeostasis.

As one might expect, the contributions of both the M1- and M2-like phenotypes to the progression of OA are distinct. To evaluate the direct effect of macrophage phenotype on osteoarthritic cartilage, cartilage explants from patients with OA were cultured in medium conditioned by macrophages stimulated by IFN-γ and TNF-α (pro-inflammatory), IL-4 (pro-healing), or IL-10 (pro-healing) (Utomo et al. 2016). In cartilage cultured in pro-inflammatory medium, IL-1β, IL-6, MMP-13, and ADAMTS5 were found to be upregulated, while aggrecan and type II collagen synthesis were inhibited. When medium conditioned by anti-inflammatory macrophages was added, gene expression levels were unaffected, suggesting M1-like macrophages directly influence cartilage degradation while M2-like macrophages do not.

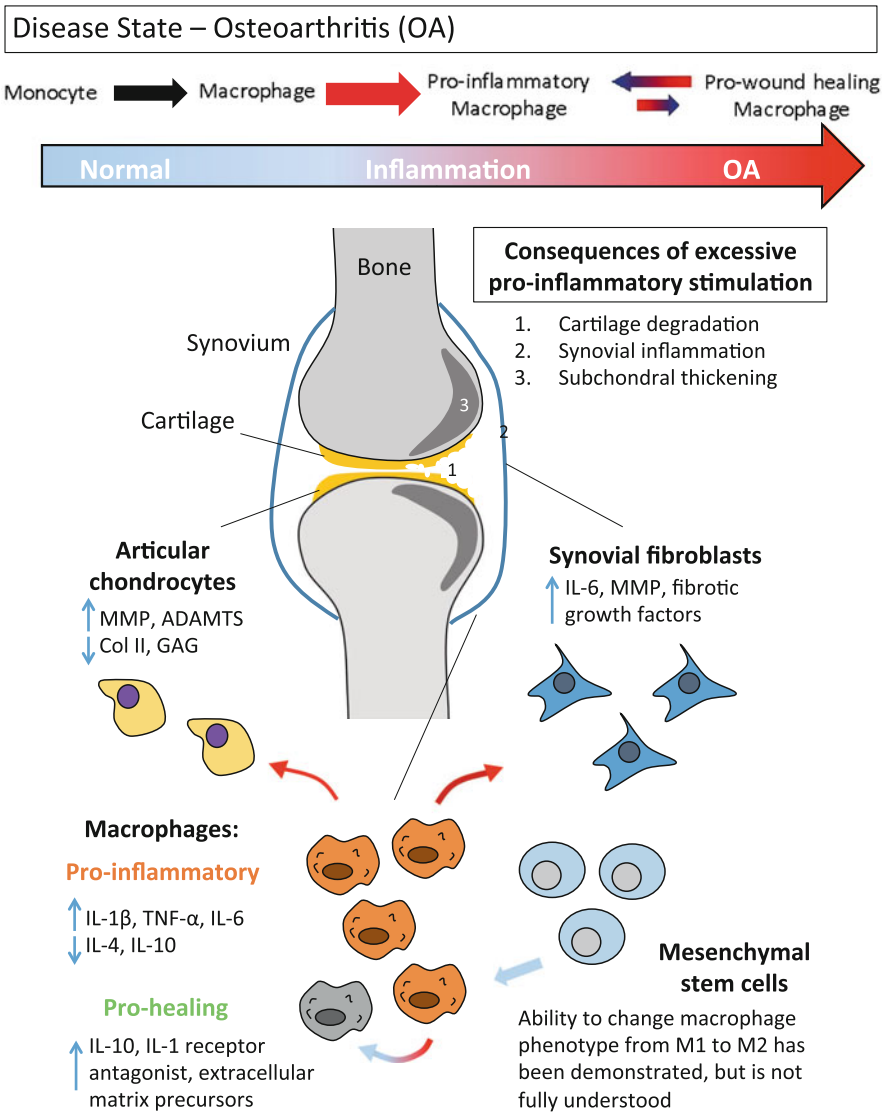

Fig. 10.4 In osteoarthritis, the regulation of macrophage phenotype, and consequently, cytokine release, is altered, leading to a disproportionate level of pro-inflammatory activity in not only macrophages but also chondrocytes and fibroblasts. Stem cells have the potential to modulate macrophage activity and are being investigated as a basis for treatment of inflammatory diseases. Art adapted from Servier Medical Arts

Macrophages also play a central role in the pathogenesis of rheumatoid arthritis (RA), although the precise conditions and causal factors leading to RA are complex and still under investigation (Davignon et al. 2013; Kinne et al. 2007). Whereas the chronic inflammation in OA is linked strongly to mechanical loading and a

pathological remodeling response, the degeneration observed in RA is representative of an autoimmune disorder not necessarily limited to connective tissue in joints and more likely to develop in genetically susceptible individuals. Manifestations of the disease bear similarities to OA, including several hallmarks: elevated macrophage numbers in the affected joint, especially in the synovium, widespread synovitis, degradation of articular cartilage, and dysregulation of inflammatory cytokines. Gene profiling via flow cytometry of $CD14^+$ monocytes isolated from synovial fluid from RA patients has indicated polarization to an M1-like phenotype (Palacios et al. 2015).

To date, therapeutic targeting of M1 macrophages or key inflammatory effector molecules such as TNF-α has had limited clinical success because of the essential protective immune function provided by these factors. Temporarily targeting specific sub-phenotypes while leaving other cell phenotypes undisturbed and allowing the continuation of essential immunoregulatory processes may lead to better treatments of OA and RA. Recently, liposome nanoparticle vesicles carrying small interfering RNA (siRNA) designed to silence molecules involved in inflammatory signaling cascades have successfully downregulated pro-inflammatory cytokine expression. In mice with experimentally induced arthritis, weekly injections of siRNA liposomes targeting cytosolic phospholipase A2-α ($cPLA_2\alpha$), an enzyme involved in prostaglandin production, resulted in local inhibition of not only $cPLA_2\alpha$ but also TNF-α and IFN-γ expression, ultimately leading to improved histological arthritis scores (Courties et al. 2011). Similarly, inflammatory progression was hindered in mice in which CCR2 (C-C chemokine receptor type 2) mRNAs were silenced via siRNA-carrying liposomes by significantly reducing the recruitment of inflammatory monocytes (Leuschner et al. 2011). CSF-1 receptor (CSF1R) may be another potential molecular target in RA therapy as treatment with anti-CSF1R antibodies improved histological scores in a murine collagen-induced arthritis model (Garcia et al. 2016). Although the efficacy of targeting specific inflammatory effectors must be demonstrated in human subjects, these therapeutic strategies show promise in developing novel OA and RA treatments.

Macrophages play a similar role in other musculoskeletal diseases. In the degenerating intervertebral disc (IVD), a similar breakdown in regulation of inflammatory mediators occurs (Freemont 2009). Degenerated IVD explants were found to have elevated levels of IL-6, IL-12, and resident macrophages (Shamji et al. 2010), directly implicating each in the progression of pathologic disc degeneration. In tendons, tendinopathic tissues exhibit elevated numbers of macrophages (Kragsnaes et al. 2014). In addition, tendon stromal cells taken from patients with supraspinatus tendon disease showed greater expression of interferon (IFN) and NF-κB target genes compared to cells taken from healthy controls (Dakin et al. 2015), indicating an amplification of inflammatory signals. In addition, in patients with early-stage tendon disease, a greater number of $CD14^+$ monocytes were found compared to intermediate-to-advanced-stage tendinopathy, which featured more $CD68^+$ macrophages, suggesting an active recruitment of pro-inflammatory monocytes that ultimately take up permanent residence as macrophages. Degenerative changes can also alter normal biology of the fibrocartilaginous knee meniscus. Degenerated

meniscus explants from aged vervet monkeys showed increased levels of key macrophage secretions including MMP, IL-6, and IL-8, although substantial levels of IL-6 and IL-8 were found in younger, healthy animals as well (Stone et al. 2015).

10.5.2 Macrophages in Musculoskeletal Soft Tissue Repair

Macrophages are also central to the natural repair of musculoskeletal soft tissues. In addition to their immune protection duties, they phagocytose both necrotic and apoptotic cellular debris generated during tissue remodeling, recycling important molecular components to be reused by the host. Excessive inflammatory activity can lead to pathological states, however. The characteristics and activity of macrophages in repair of joint capsule tissues including articular cartilage, synovium, and the meniscus in the knee can be modulated by introducing stem cells into the damaged region. Several studies have demonstrated the potential of stem cells as therapeutic vehicles to directly influence macrophage cytokine secretion and ultimate structure of new ECM. Conditioned medium from human mesenchymal stem cell (MSC) spheroid culture was shown to produce anti-inflammatory, M2-like behavior in macrophages polarized by exposure to bacterial LPS (Ylostalo et al. 2012). The secretion of pro-inflammatory mediators such as TNF-α was inhibited, while the expression of anti-inflammatory IL-10 and IL-1 receptor antagonist was increased. When bone marrow-derived MSC were co-cultured with anti-inflammatory macrophages in collagen scaffolds, GAG production increased compared to a co-culture of MSC with pro-inflammatory macrophages (Sesia et al. 2015). As natural healing in soft tissues such as the meniscus or articular cartilage is minimal due to limited vasculature, stem cell-based repair strategies show promise by alleviating macrophage-induced inflammation.

Tendon and ligament repair may also benefit from macrophage modulation. A number of studies have focused on the ultimate tensile properties of healing tendon or ligament. One such study involving nonspecific macrophage-ablation treatment via liposome-encapsulated bisphosphonate following anterior cruciate ligament (ACL) reconstruction in rats found increased tensile strength and stiffness of the tissue at the healed tendon-bone interface (Hays et al. 2008). Consistent with the hypothesis that the absence of macrophages leads to improved mechanical strength of the healed tissue, findings from an Achilles tendon injury model in macrophage-depleted mice suggest that the presence of invading macrophages may accelerate the healing response but that ultimate tensile properties of the healed tendon are inferior (de la Durantaye et al. 2014). Despite the adverse effects of excessive pro-inflammatory stimulation, however, early macrophage infiltration and cytokine secretion may be required to initiate a normal response to tissue damage. In a rat medial collateral ligament (MCL) injury model, macrophage-depleted animals displayed reduced numbers of both M1 and M2 phenotypes and lower mechanical strength after healing. Furthermore, macrophage numbers returned to control levels after 5 days, after which a wound healing response was mounted (Chamberlain

et al. 2011a, b). Co-cultures of adipose-derived stem cells (ASC) with pro-inflammatory macrophages isolated from mice tail tendons showed an increase in macrophage expression of CD206, a marker specific to pro-healing phenotypes, effectively demonstrating a switch from M1 to M2, preventing inflammation progression in vitro (Manning et al. 2015). Interestingly, MSCs "primed" to display more anti-inflammatory behavior were shown to have an enhanced benefit in a rat MCL injury model (Saether et al. 2016). The ligament that healed in the presence of primed MSC displayed higher IL-1 receptor antagonist and procollagen levels as well as greater M2 macrophage numbers compared to ligament that healed with unprimed MSC.

Molecular factors have also been studied as modulators of macrophage activity in tendon and ligament healing. In a rat MCL injury model, administration of IL-4, an anti-inflammatory factor, before and after ligament rupture decreased M1 macrophage numbers, wound size, and type III collagen levels while increasing procollagen I formation (Chamberlain et al. 2011a, b). Unfortunately, administration of IL-4 alone was unable to sustain these effects beyond 5 days or affect ultimate ligament strength. The contribution to tendon healing of factors with opposite inflammatory roles, IL-4 (anti-inflammatory) and IL-6 (pro-inflammatory), was investigated in a tendon injury model in transgenic IL-4 and IL-6 knockout mice (Lin et al. 2006). Contrary to the hypothesis that a lack of IL-6 would increase mechanical strength and collagenous organization by decreasing inflammation and that a lack of IL-4 would decrease strength and organization by increasing inflammation, IL-6-deficient mice exhibited lower strength, while IL-4-defiicent mice displayed mechanical properties comparable to uninjured controls. It was reasoned that the absence of IL-6 prompted an inadequate inflammatory response, while other effector molecules were able to compensate for the absence of IL-4. Neither macrophage numbers nor polarization was evaluated, however. Elucidating the interactions between stem cells and inflammatory cells and molecules may lead to improved tendon and ligament repair strategies.

10.6 Conclusion and Future Directions

In this chapter, we described the role of macrophages in specific cases of injury and disease. Interestingly, the ways in which macrophages are altered are disease and injury specific. After myocardial infarction, increased numbers of M1-like and M2-like macrophages are involved in an ongoing remodeling process. In volumetric muscle loss, the decrease in population of progenitor cells and M1-like macrophages results in an impaired repair process leading to less functional tissue. Finally, osteoarthritis is a long-term degenerative process that involves increased levels of M1-like macrophages over a long time period. Given the large number of people impacted by these diseases, an increased understanding of the inflammatory process, specifically the role of macrophages, could lead to improved treatment.

In the future, an improved definition of macrophage phenotypes will benefit the understanding of natural healing and disease processes. As an example, it is apparent that a complex interplay exists between multiple macrophage phenotypes. Both M1-like and M2-like macrophages are required for normal tissue healing. Although the levels of M1-like and M2-like macrophages may be out of balance during disease and injury, ablation of all macrophages or only M1-like macrophages leads to inferior results. How the level and balance of polarized macrophage is maintained and how the balance of these cells influence other cells, such as stem cells, are unknown but deserving of continued study. Moreover, by harnessing the complex role of macrophages, enhanced therapeutic treatments can be developed that enhance the normal healing response as well as help the survival of therapeutic cells delivered to the site of injury.

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Chapter 11
Macrophages and Their Contribution to the Development of Atherosclerosis

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Abstract Atherosclerosis can be regarded as chronic inflammatory disease driven by lipid accumulation in the arterial wall. Macrophages play a key role in the development of local inflammatory response and atherosclerotic lesion growth. Atherosclerotic plaque is a complex microenvironment, in which different subsets of macrophages coexist executing distinct, although in some cases overlapping functions. According to the classical simplified nomenclature, lesion macrophages can belong to pro-inflammatory or anti-inflammatory or alternatively activated types. While the former promote the inflammatory response and participate in lipid accumulation, the latter are responsible for the inflammation resolution and plaque stabilisation. Atherosclerotic lesion dynamics depends therefore on the balance between these macrophages populations. The diverse functions of macrophages make them an attractive therapeutic target for the development of novel anti-atherosclerotic treatments. In this chapter, we discuss different types of

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macrophages and their roles in atherosclerotic lesion dynamics and describe the results of several experiments studying macrophage polarisation in atherosclerosis.

11.1 Introduction

Atherosclerosis and related cardiovascular diseases remain the leading cause of morbidity and mortality worldwide (Sanchis-Gomar et al. 2016). Despite the considerable progress during the recent years, treatment and prevention of atherosclerosis are still a challenge for the modern medicine, partly because of the complex nature of the disease pathogenesis. It is currently established that atherosclerotic lesion development is dependent on several processes. First, altered blood lipoprotein profile with prevailing low-density lipoprotein (LDL) and especially its modified forms facilitates lipid accumulation in the arterial wall (Krauss 2010). Second, endothelial dysfunction allows lipoprotein particles penetrating into the subendothelial layer of the arterial wall (intima), where the plaque development takes place (Gimbrone and García-Cardeña 2016). Third, local inflammatory response and misbalanced functioning of tissue macrophages contribute to the plaque growth and the formation of lipid core, inside which necrotic processes can take place (Libby 2013). Although growing atherosclerotic plaques may reduce the vessel volume, they often remain asymptomatic for years, which hinder the timely diagnostics of the condition. At later stages, atherosclerotic plaques can acquire a fibrous cap, which separates them from the vessel milieu and renders them stable. The primary danger comes from the so-called unstable plaques, as they are likely to induce thrombus formation on the surface (Hansson et al. 2015). This process can have a serious or fatal outcome, inducing thromboembolism of vital organs. For many patients, this serious event would be the first clinical manifestation of atherosclerosis.

Inflammation plays a central role at all stages of atherosclerosis development. In fact, atherosclerosis is currently regarded as a chronic inflammatory condition driven by misbalance of plasma lipoprotein profile and other factors (Ross 1999; Libby 2002). Importantly, inflammation and the imbalanced macrophage function are likely to play a decisive role in the formation of unstable plaques and plaque rupture followed by life-threatening thrombus formation. Therefore, studying inflammatory processes associated with atherosclerosis became a hot research topic during recent years.

The development of atherosclerotic lesion is associated with a local inflammatory response with activation of various cell types, including monocytes/macrophages (Moore and Tabas 2011). Monocytes are recruited to the lesion site, where they differentiate into macrophages and actively participate in lipoprotein particle uptake. The increase of the tissue population of macrophages can result not only from the recruitment of monocytes from circulation but also from macrophage proliferation in the tissue (Ginhoux and Jung 2014). In advanced plaques, proliferation accounts for the largest part of the macrophage population increase (Robbins et al. 2013; Orekhov et al. 2010). Intracellular lipid accumulation by macrophages

leads to the formation of foam cells that have cytoplasm filled with lipid droplets. Such cells are abundant in progressing atherosclerotic plaques. Interestingly, the subendothelial layer of arterial intima contains a population of pluripotent cells also known as macrovascular pericytes, which have a capacity for phagocytosis, express macrophage marker CD68 and can also become foam cells (Orekhov et al. 2014). Foam cells secrete various signalling molecules contributing to the formation of atherosclerotic plaque microenvironment rich in pro-inflammatory cytokines and factors (Libby 2002). This promotes further recruitment of circulating monocytes, lipoprotein retention and extracellular matrix remodelling. Macrophages populating atherosclerotic plaques have a decreased ability to migrate (Randolph 2014). Failure to remove dying macrophages in the lipid core of the plaque leads to the formation of necrotic area (Seimon and Tabas 2009). In advanced plaques, where neovascularisation has taken place, macrophages are responsible for clearance of erythrocytes that enter the plaque following ruptures of blood vessels (Kockx et al. 2003). These processes further contribute to the lesion progression and the development of complicated plaques.

As can be seen from this brief overview, macrophages represent an important component of the pathogenesis of atherosclerosis and a potential point of therapeutic intervention. Several macrophage-targeting strategies have been proposed, such as inhibiting monocyte recruitment to the lesion site, stimulating cholesterol efflux and diminishing lipid storage in macrophages and modulating macrophage polarisation towards pro- or anti-inflammatory phenotypes (Moore et al. 2013). However, the development of macrophage-targeting therapy is complicated by macrophage heterogeneity and plasticity that necessitate a well-balanced approach.

11.2 Reticuloendothelial System

Monocytes and macrophages are regarded as a continuous system, also known as mononuclear phagocyte system, which plays a central role in the innate immune response (van Furth and Cohn 1968). Monocytes circulate in the bloodstream and can differentiate to macrophages in response to various signals, such as tissue injury and pathogen invasion that induce secretion of cytokines and chemokines by tissue cells. Monocyte-derived macrophages are capable of active phagocytosis and produce pro-inflammatory factors that orchestrate the immune response to pathogens. The results of early experiments with radiolabelled monocytes in animal models suggested that all macrophages at the lesion site derive from the recruited monocytes and represent a terminally differentiated cellular population. This view, however, has been challenged by more recent studies that have demonstrated that, at least in some cases, tissue macrophage population can expand by proliferation (Ginhoux and Jung 2014). Importantly, macrophage proliferation has been shown to contribute to atherosclerotic lesion growth (Orekhov et al. 2010).

11.2.1 Macrophage Heterogeneity

The population of macrophages is characterised by heterogeneity: several macrophage types can be distinguished based on the gene expression pattern and main functions. Studying of macrophage differentiation and heterogeneity is challenging because activation of cells can occur during the isolation process, and this can influence the obtained macrophage population properties. Moreover, macrophage subtypes described in animal models, such as mice, do not coincide fully with the subtypes present in humans, which makes the research even more complicated. In the classical model of macrophage activation, two main phenotypes have been defined mirroring the two types of T helper cells (Th1 and Th2): pro-inflammatory classically activated (M1) and alternatively activated (M2) (Mantovani et al. 2002). Later, however, the accumulating knowledge made it evident that the classical model should be revised to describe the macrophage complexity more accurately (Murray et al. 2014; Martinez and Gordon 2015). For accurate study of macrophage heterogeneity, it is currently recommended to define the macrophage subtypes based on several established markers and also the activation stimuli that triggered their differentiation. For experimental purposes, the classical model still can be used as a tool, since it is relatively simple and allows distinguishing and modelling major imbalances between pro- and anti-inflammatory macrophages in pathological conditions (de Gaetano et al. 2016; Novoselov et al. 2015).

11.2.2 A Simplified Classification: Pro- and Anti-inflammatory Macrophages

Pro-inflammatory M1 macrophages can be induced in response to Th1 cytokine interferon (IFN)-γ, as well as pathogen-associated molecular complexes (PAMPs), lipopolysaccharides and lipoproteins. In the context of atherosclerotic plaque development, M1 macrophage polarisation involves NF-κB and NLRP3 inflammasome pathways (Duewell et al. 2010). M1 macrophages produce pro-inflammatory factors, such as tumour necrosis factor (TNF)-α, interleukin-1β (IL-1β), IL-6, IL-12, IL-23 and Th1 cell-attracting chemokines CXCL9, CXCL10 and CXCL11, as well as reactive oxygen species (ROS) and nitric oxide (NO). Therefore, M1 macrophages stimulate and maintain the inflammatory response.

Alternatively activated M2 macrophages are induced in response to Th2-type cytokines IL-4, IL-33 and IL-13. They secrete anti-inflammatory factors, such as IL1 receptor agonist (IL-1RA) receptor agonist and IL-10 and chemokines CCL17, CCL22 and CCL24 (Martinez et al. 2006). M2 macrophages are responsible for inflammation resolution, tissue repair and remodelling. These cells are characterised by high phagocytic activity and expression of a variety of scavenger receptors. Within the group of alternatively activated macrophage, several subtypes can be distinguished based on the activation stimuli and gene expression pattern. For

instance, M2a macrophages can be induced by IL-4 and IL-13 and express high levels of CD206 and IL-1 receptor agonist. M2b macrophages are induced by TLR signalling, immune complexes and IL-1R ligands and produce both pro- and anti-inflammatory cytokines, such as IL-10, IL-6 and TNF-α (Martinez et al. 2008). M2c macrophages can be induced by IL-10, transforming growth factor-β (TGF-β) and glucocorticosteroids and have strong anti-inflammatory properties, producing pentraxin-3 (PTX3), TGF-β and IL-10 (Zizzo et al. 2012). M2d macrophages are induced by TLR signalling and have angiogenic properties, playing a role in tumour progression and atherosclerotic plaque growth (Ferrante et al. 2013).

More macrophage varieties have been described in experimental conditions and discovered in vivo. The picture becomes even more complex after taking into account macrophage varieties that are induced in some pathological conditions and are clearly distinct from the classical M1 and M2 macrophages. For instance, activation with oxidised phospholipids can result in the formation of distinct macrophage phenotype Mox expressing redox regulatory genes that can be found in large quantities in atherosclerotic lesions in mouse models (Kadl et al. 2010). A shift in macrophage phenotypes and formation of mixed phenotypes has been observed in obesity, cancer and other pathological conditions (Biswas and Mantovani 2010).

11.3 Macrophages and Atherosclerosis

11.3.1 Adhesion and Penetration of Monocytes into the Arterial Wall

According to the current understanding, circulating monocytes belong to one of the several distinct subtypes described in humans and mice. These subtypes are characterised by the expression of certain surface markers and chemokine receptors (Geissmann et al. 2003). In humans, monocytes that express CD14 and CC-chemokine receptor 2 (CCR2) and are negative for CD16 surface antigen are the most prevalent and referred to as classical monocytes (Ziegler-Heitbrock 2007; Ziegler-Heitbrock et al. 2010). Monocytes that are positive for CD16 can be further divided into two subsets: $CD14^{+}CD16^{++}$ (non-classical) monocytes that perform patrolling and $CD14^{++}CD16^{+}$ (intermediate) that have pro-inflammatory properties (Cros et al. 2010; Belge et al. 2002). Interestingly, increased numbers of pro-inflammatory monocytes have been demonstrated in animal models of atherosclerosis (*Apoe*$^{-/-}$ mice) (Swirski et al. 2007). The relationship between monocyte predisposition to the inflammatory response and other cardiovascular risks remains to be elucidated in full detail. However, it has been demonstrated that hypercholesterolaemia resulted in enhanced proliferation of haematopoietic stem cells and their sensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF), which triggers macrophage polarisation towards the pro-inflammatory phenotype. On the other hand, the expression of high-density lipoprotein (HDL),

which has protective properties against atherosclerosis development, reversed this phenotype (Yvan-Charvet et al. 2010).

Atherosclerotic lesion formation begins with local endothelial dysfunction with increased permeability of the endothelial lining and establishment of pro-inflammatory microenvironment. This process is known as endothelial activation (Pober and Sessa 2007). This process can be induced by a number of factors, including modified LDL, lipopolysaccharides and cytokines, such as TNF-α and interleukin (IL)-β. Endothelial activation has been demonstrated to be mediated by nuclear factor-κB (NF-κB) signalling (Collins and Cybulsky 2001). This induces the expression of cell adhesion molecules on the endothelium that facilitate the recruitment of circulating immune cells: intercellular adhesion molecule-1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1), E-selectin, such as MCP1, platelet-derived growth factor (PDGF). In animal models, it has been demonstrated that both pro-inflammatory and patrolling monocytes can be recruited to growing atherosclerotic lesions by P- and E-selectin-dependent rolling followed by ICAM1- and VCAM1-dependent adhesion (Galkina and Ley 2007). Migration of pro-inflammatory monocytes into the arterial wall is dependent on CCR2, CCR5 and CX_3C-chemokine receptor 1 (CX_3CR1) signalling, and inhibition of these pathways had a protective effect in *Apoe*$^{-/-}$ mice reducing atherosclerotic plaque growth (Combadiere et al. 2008).

At the lesion site, monocyte-derived macrophages can take part in different processes: pro-inflammatory cells contribute to the inflammatory response and lesion development, while patrolling monocytes may participate in phagocytosis or differentiate into dendritic cells (Swirski et al. 2007, 2009). Dendritic cells play a prominent role in atherosclerotic plaque development, and their functions overlap to some extent with those of macrophages (Cybulsky et al. 2016). Differentiation of macrophages is accompanied by morphological changes, such as enlargement, increase of the number of organelles and alteration of gene expression patterns, which increases their sensitivity to signalling molecules. Intensification of the lysosomal enzyme activity prepares the cells to active phagocytosis (Novoselov et al. 2015). Importantly, differentiated macrophages in atherosclerotic plaques have a decreased ability to migrate, which hinders the inflammation resolution and favours plaque growth.

11.3.2 Pro- and Anti-inflammatory Macrophages in Atherosclerotic Lesions

Developing atherosclerotic lesion is a specific environment, which is enriched in activated cells, pro-inflammatory factors and modified lipoproteins. At later stages, plaques contain large quantities of dying and apoptotic cells that are subject to macrophage-mediated clearance. Both pro- and anti-inflammatory phenotypes have been found in atherosclerotic lesions at different stages (De Paoli et al. 2014;

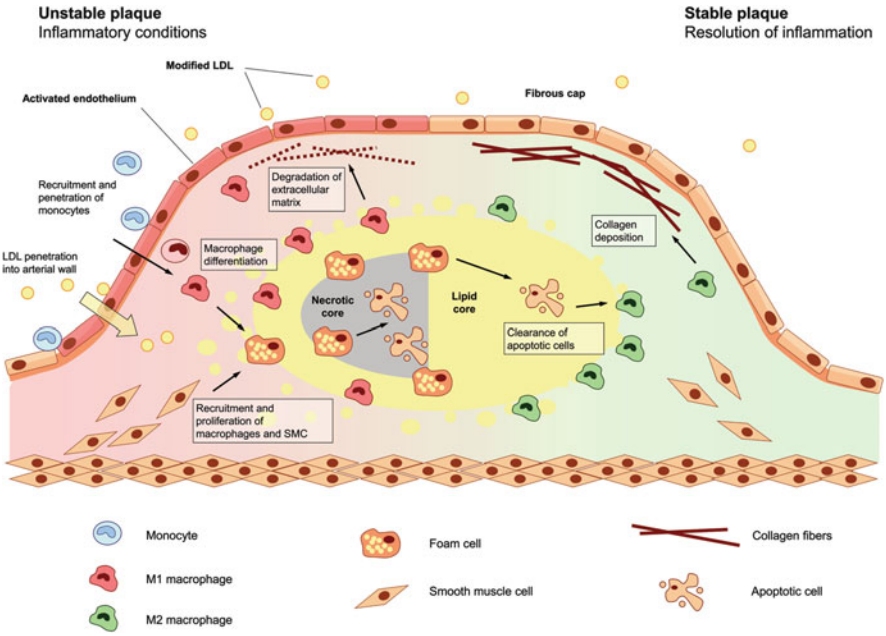

Fig. 11.1 Simplified scheme of the roles of pro- and anti-inflammatory macrophages in atherosclerotic lesion progression. Pro-inflammatory conditions are associated with high plasma concentrations of atherogenic lipoprotein and local endothelial dysfunction and activation. Monocyte recruitment and infiltration into arterial wall is facilitated in such conditions, and pro-inflammatory (M1) macrophage polarisation is stimulated. M1 macrophages participate in lipid accumulation, maintain pro-inflammatory environment and promote plaque destabilisation. Alternatively activated M2 macrophages contribute to plaque stabilisation via inflammation resolution, efferocytosis of dying cells and stimulation of extracellular matrix synthesis

Bouhlel et al. 2007), and their role in the disease pathogenesis appears to be complex (Fig. 11.1). On one hand, pro-inflammatory (M1) macrophages have long been known as a major factor promoting the local inflammation and plaque growth (Smith et al. 1995). On the other hand, alternatively activated macrophages are also found at the lesion sites and are important for inflammation resolution and atherosclerotic plaque regression (Nathan and Ding 2010). Both pro- and anti-inflammatory macrophage populations increase in course of atherosclerotic lesion progression, but their distribution within the plaque is not identical. Immunocytochemistry studies demonstrated that cells positive for M1 markers are preferentially found in the plaque shoulder regions, and cells, positive for M2 markers—in the adventitia (Stoger et al. 2012). Interestingly, prevalence of M2 or M1 macrophages was demonstrated to be a hallmark of plaque stability or instability correspondingly. Macrophages positive for M2 markers surface mannose receptor (MR) and CD68 were found in more stable regions of plaques that were also enriched with IL-4. These cells were apparently more resistant to foam cell formation, as MR-positive (M2) macrophages contained fewer and smaller lipid droplets

compared to MR-negative macrophages (Chinetti-Gbaguidi et al. 2011). Studies evaluating macrophage population in symptomatic versus asymptomatic plaque specimens obtained in course of carotid endarterectomy demonstrated an increased content of M1 marker-expressing and decreased content of M2 marker-expressing macrophages in symptomatic plaques (Cho et al. 2013). In contrast, asymptomatic lesions predominantly contained cells positive for M2 markers, such as CD163. This observation was further confirmed in a more recent study employing a panel of specific M1 and M2 markers (de Gaetano et al. 2016).

Haemorrhagic lesions develop at later stages of atherosclerotic plaque progression. Erythrocyte contamination is cleared by macrophages that are characterised by a distinct gene expression pattern. So-called HA-mac population expresses high levels of CD163 and low levels of human leukocyte antigen-DR and is resistant to oxidative stress, which allows these cells to clear iron-containing haem more efficiently (Boyle et al. 2009). These macrophages are likely to play a protective role reducing oxidative stress. Haem-induced macrophage phenotype (also called Mhem) was demonstrated to be resistant to lipid accumulation and foam cell formation through activating transcription factor 1 (ATF-1) signalling, which induces liver X receptor-β (LXR-β) leading to the induction of genes coordinating cholesterol efflux, such as LXR-α and ABCA1 (Boyle et al. 2012). This has also been demonstrated on cultured human monocytes exposed to haemoglobin:haptoglobin complexes, which led to formation of a distinct M(Hb) macrophage phenotype positive for MR and CD163 and resistant to foam cell formation (Finn et al. 2012).

Another macrophage phenotype present in atherosclerotic lesions is M4, a distinct phenotype, which can be induced by CXCL-4 chemokine. M4 macrophages are characterised by simultaneous expression of matrix metalloproteinase (MMP)7 and calcium-binding protein S100A8. They express pro-inflammatory cytokines IL-6 and TNF-α and are negative for CD163 (Erbel et al. 2015). These macrophages can be considered pro-atherogenic, since they may promote destabilisation of the plaque fibrous cap (Chistiakov et al. 2015).

11.3.3 Lipid Metabolism and Accumulation by Macrophages

According to the current understanding, LDL serves as the primary source of lipid accumulation in the arterial wall during atherosclerotic plaque development. However, the accumulating evidence demonstrates that only certain types of LDL that underwent atherogenic modification are associated with the increased risk of atherosclerosis. Native (non-modified) LDL particles are recognised by LDL receptor (LDLR) and internalised by cells via receptor-mediated endocytosis, which is a highly regulated process. Particles internalised following this pathway are transported to lysosomes and degraded by lysosomal acid hydrolases. In this process, cholesterol esters are transformed to free cholesterol, which is transported to the endoplasmic reticulum and esterified by cholesterol acyltransferase (ACAT)

(Brown and Goldstein 1983). High amounts of free cholesterol in the endoplasmic reticulum initiate a signalling cascade that decreases the expression of LDLR and subsequent lipid uptake. This regulation prevents lipid overload and foam cell formation. ApoB-containing lipoproteins that also contain ApoE can cause cholesterol accumulation through interaction with ApoE receptors, such as LRP1 and VLDL receptors that are not regulated by the amount of intracellular cholesterol. Uptake of native LDL via pinocytosis is also possible and can lead to foam cell formation, as this process is less strictly regulated (Kruth 2011). However, in atherosclerotic lesions, massive lipid uptake is likely to occur in process of uncontrolled phagocytosis (Torzewski and Lackner 2006; Torzewski et al. 2004). This internalisation pathway is taken by various LDL-containing aggregates that are especially atherogenic. Interestingly, modified LDL, such as desialylated LDL, is susceptible to aggregation and can also induce formation of autoantibodies followed by LDL-containing immune complexes (Sobenin et al. 2014).

Modified LDL, such as oxLDL, can be internalised by cells via scavenger receptors that are not regulated by intracellular cholesterol levels, including CD36, scavenger receptor A (SRA), lectin-like receptors (LOX) and toll-like receptors (TLRs) (Moore and Freeman 2006; Younis et al. 2008). The role of modified LDL (mLDL) in the pathogenesis of atherosclerosis has been widely studied during the last years. Converging evidence indicates that LDL particles undergo multiple modifications in the bloodstream, including desialylation, decrease of particle size and increase of density, acquisition of negative charge and oxidation (Tertov et al. 1998). Desialylated LDL could be isolated from blood plasma of patients with confirmed atherosclerosis and used for modelling lipid accumulation in human arterial wall cells in primary culture. Experiments on cultured macrophages demonstrated that atherogenic mLDL caused a substantial increase of the expression of both pro-inflammatory marker TNF-α and anti-inflammatory CCL18, while native, unmodified LDL did not possess such activity (Tables 11.1 and 11.2). These results indicate that cholesterol accumulation can result in significant alteration of macrophage gene expression involving pro- and

Table 11.1 Effect of LDL on cytokine gene expression

	Native LDL	Atherogenic LDL
TNF-α	1.0 ± 0.3	2.1 ± 0.5[a]
CCL18	18.1 ± 6.4	27.8 ± 9.6[a]

Monocytes were isolated from whole blood of healthy donors by density gradient followed by selection of CD14+ cells by magnetic separation. Cells were cultured for 7 days. Native or atherogenic LDL was added at a concentration of 100 μg/ml and the cells were incubated for 24 h. RNA was isolated and gene expression was measured by RT-PCR technique. The figures show the relative expression of the genes TNF-α (n = 14) and CCL18 (n = 7). Control gene expression in untreated cells was taken as reference (1)

[a]Significant differences from native LDL (Student's T-test)

Table 11.2 Ontology of genes whose activity changes in the accumulation of intracellular cholesterol by human macrophages[a]

Term	Genes
Sterol transporter activity (GO:0015248)	ABCA1;APOE;ABCG1
Cholesterol transporter activity (GO:0017127)	ABCA1;APOE;ABCG1
Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor (GO:0016628)	AKR1C1;FASN;AKR1C3
Steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (GO:0033764)	HSD3B7;AKR1C1;AKR1C3
Steroid dehydrogenase activity (GO:0016229)	HSD3B7;AKR1C1;AKR1C3
Cholesterol binding (GO:0015485)	ABCA1;NR1H3;ABCG1
Protein homodimerisation activity (GO:0042803)	G6PD;SLC11A1;FASN;PDGFC;MGST1; ABAT;APOE;GLA;ABCD1;ABCG1; SYNE1
Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (GO:0016616)	HSD3B7;G6PD;AKR1C1;FASN;AKR1C3
Oxidoreductase activity, acting on CH-OH group of donors (GO:0016614)	HSD3B7;G6PD;FASN;AKR1C1;AKR1C3
Steroid binding (GO:0005496)	ABCA1;AKR1C1;NR1H3;ABCG1
Sterol binding (GO:0032934)	ABCA1;NR1H3;ABCG1
Lipid transporter activity (GO:0005319)	ABCA1;APOE;ABCD1;ABCG1
Alcohol binding (GO:0043178)	ABCA1;PLCL1;NR1H3;ABCG1
Aldo-keto reductase (NADP) activity (GO:0004033)	AKR1C1;AKR1C3
Alcohol dehydrogenase (NADP+) activity (GO:0008106)	AKR1C1;AKR1C3
Solute:proton antiporter activity (GO:0015299)	SLC11A1;SLC9A9
Active transmembrane transporter activity (GO:0022804)	SLC2A9;SLC11A1;SLC9A9;SLC16A7; ABCD1;ABCG1
Cation:cation antiporter activity (GO:0015491)	SLC11A1;SLC9A9
Oxidoreductase activity, acting on the CH-CH group of donors (GO:0016627)	AKR1C1;FASN;AKR1C3
Cargo receptor activity (GO:0038024)	CD163;TFRC;STAB1

[a]Monocytes were isolated from whole blood of healthy donors by density gradient. Cells were cultured for 7 days. Native, oxidised or accetylated LDL was added at a concentration of 100 μg/ml and the cells were incubated for 24 h. RNA was isolated and transcriptome analysis was performed. Gene ontology was obtained by EnrichR (Chen et al. 2013; Kuleshov et al. 2016)

anti-inflammatory factors, which has also been demonstrated in previous studies (De Paoli et al. 2014; Hägg et al. 2009).

At the immunohistochemical level of investigation, CD68 which is mainly present in macrophage lysosomes represents a reliable marker for the identification of macrophages and macrophage origin foam cells in atherosclerotic lesions (Bobryshev et al. 2013) (Fig. 11.2a). Ultrastructural analysis has shown that the

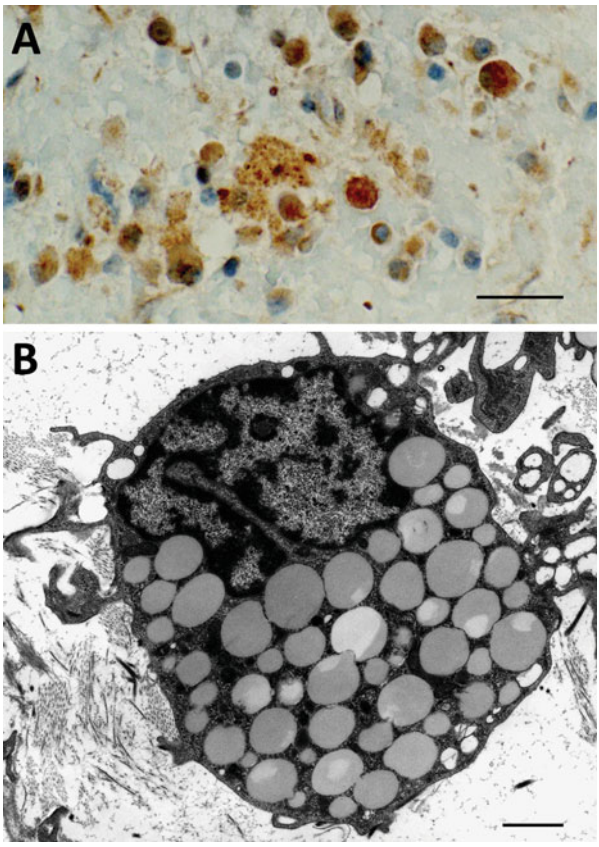

Fig. 11.2 Formation of foam cells in atherosclerotic lesions (**a**, **b**). (**a**) Macrophages and foam cells of macrophage origin located in an atherosclerotic lesion, identified using anti-CD68 antibody. Immunohistochemistry; peroxidase–anti-peroxidase technique. (**b**) Accumulation of a large number of lipid inclusion ("lipid droplets") in the cytoplasm of a macrophage, leading to the formation of a foam cell. Transmission Electron Microscopy. Scale bars = 25 μm (**a**) and 2 μm (**b**)

formation of foam cells occurs as a result of the accumulation of a large number of lipid inclusions (so-called "lipid droplets") in the cytoplasm of macrophages (Bobryshev 2006) (Fig. 11.2b). In vitro experiments, in which macrophages were incubated with modified low-density lipoproteins (LDL), showed that modified lipoproteins are captured by macrophages (Nagornev et al. 1985, 1991) (Fig. 11.3). It is commonly accepted that similar unregulated capture of modified LDL occurs in atherosclerotic lesions in situ as well and that this process is responsible for the formation of foam cells in the arterial wall (Nagornev et al. 1991; Ross 1999). The process of the formation of foam cells is tightly associated with functioning of lysosomes in macrophages, more exactly to say—with the inability of lysosomes to completely catabolise modified LDL that are captured by macrophages from the extracellular space (Nagornev et al. 1991; Bobryshev et al. 2013). As a morphological evidence of such incomplete catabolism of lipids in lysosomes, the appearance of secondary lysosomes/autophagosomes containing lipid inclusions can be considered (Figs. 11.4 and 11.5).

From a biochemical point of view, inside the cell, cholesteryl esters accumulate in cytoplasmic droplets, where neutral cholesterol esterase hydrolyses them to free

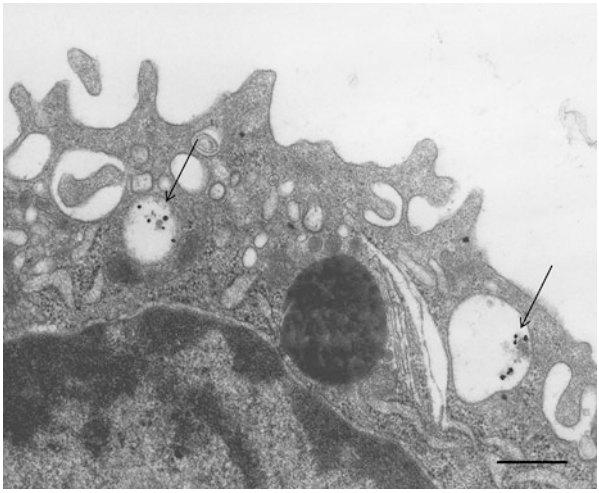

Fig. 11.3 Accumulation of oxidised low-density lipoproteins (oxLDL) labelled with gold particles (*arrows*) in endocytotic vacuoles in the cytoplasm of a macrophage, revealed in an in vitro experiment. Transmission Electron Microscopy. Scale bar = 200 nm

cholesterol that, in turn, is esterified by ACAT. Accumulation of free cholesterol induces pro-inflammatory activation of macrophages resulting in the endoplasmic reticulum stress (Li et al. 2005) and calcium leak into the cytosol (Lim et al. 2008). Accumulation of lipid droplets in the cytoplasm was demonstrated to cause activation of TLR4, which increases lipid uptake and further promotes foam cell formation, while accumulation of cholesterol crystals induces inflammasome activation (Choi et al. 2009; Duewell et al. 2010; Tall and Yvan-Charvet 2015). TLR activation also promotes the production of pro-inflammatory factors by macrophages, including IL-1β and chemokine (C-C motif) ligand 5 (CCL5) (Bae et al. 2009). Cholesteryl esters can promote pro-inflammatory responses via different signalling pathways, including 7-ketocholesteryl-9-carboxynonanoate that was demonstrated to activate NF-κB pathway and cholesteryl linoleate–MAP kinase signalling (Huang et al. 2010; Huber et al. 2002). Comparison of macrophages laden with cholesterol with control cells demonstrated that that cholesterol accumulation can alter macrophage metabolism and response to the external stimuli. Cholesterol-laden macrophages exposed to pro-inflammatory stimulators expressed a lower level of inflammation markers than in control, but no difference could be seen in anti-inflammatory response of these cells (da Silva et al. 2016).

Oxysterol is another pro-inflammatory cholesterol derivative, which is present in atherosclerotic plaques. It has been demonstrated that oxysterol induced the expression of monocyte chemoattractant-1 (MCP-1) in macrophages (Leonarduzzi et al. 2010). In addition, exposure to oxidised cholesterol esters also enhances the expression of scavenger receptor CD36 (Jedidi et al. 2006). Therefore, exposure to atherogenic lipoprotein particles can induce pro-inflammatory phenotypes in monocytes/macrophages. Moreover, it has been demonstrated that oxLDL could shift the phenotype of alternatively activated M2 macrophages towards the pro-inflammatory through changes in gene expression (van Tits et al. 2011).

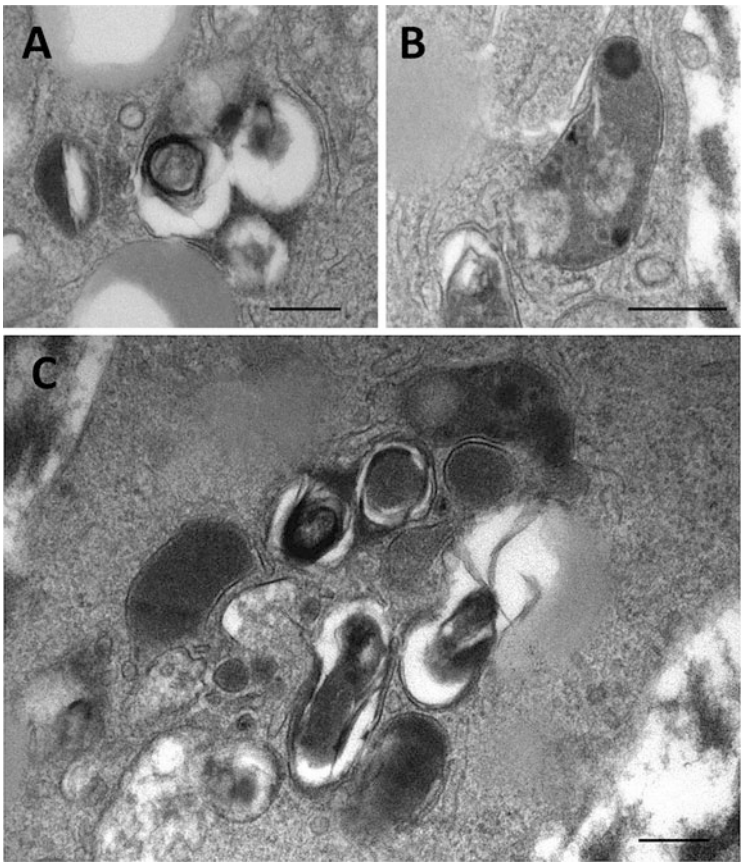

Fig. 11.4 Structural appearance of lysosomes in intimal cells containing "lipid droplets" in intimal cells in fatty streaks (Type II lesions) (**a**–**c**). Note that, while few lysosomes are characterised by the presence of homogenous material of middle-high electron density, the majority of lysosomes are represented by secondary lysosomes and autophagosomes, containing lipid inclusions. (**a**–**c**): Transmission Electron Microscopy. Scale bars = 200 nm (**a**–c). (Reproduced from Bobryshev et al. 2013; with permission from Wiley) (Bobryshev YV, Shchelkunova TA, Morozov IA, Rubtsov PM, Sobenin IA, Orekhov AN, Smirnov AN. Changes of lysosomes in the earliest stages of the development of atherosclerosis. J Cell Mol Med 2013;17 (5):626–35)

Within the acidic microenvironment of lipid plaques, phospholipase-mediated hydrolysis of lipoproteins can result in the formation of products that greatly contribute to the lipid accumulation in the arterial wall. For instance, phospholipase A-treated LDL increase the secretion of TNF-α and IL-6 by macrophages and promoted foam cell formation (Boyanovsky et al. 2010).

It has long been known that some classes of lipids, such as high-density lipoprotein (HDL) and polyunsaturated fatty acids (PUFA), have atheroprotective properties. These properties may partly be explained by their effect on macrophages. For instance, experiments on mice demonstrated that conjugated linoleic

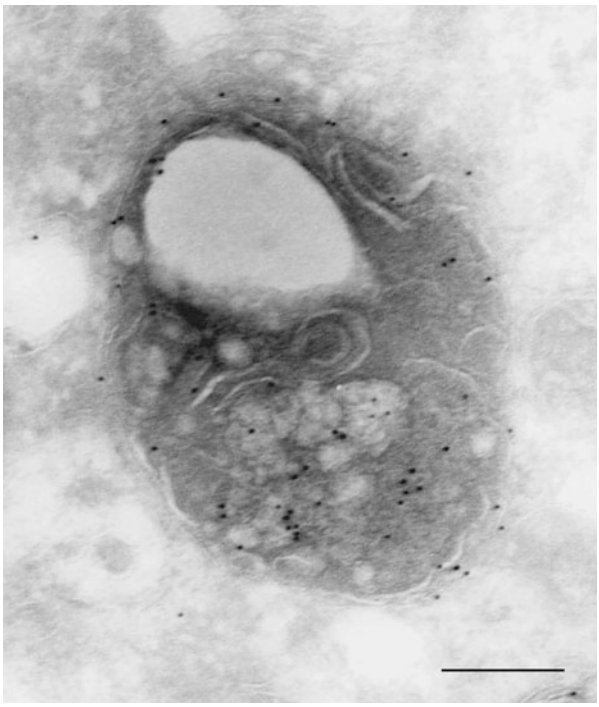

Fig. 11.5 A high-resolution micrograph showing the distribution of CD68 antigen in an autophagosome in an intimal cell in a fatty streak of the human aorta. Electron microscopic immunocytochemistry; immunogold technique. Scale bar = 200 nm. (Reproduced from Bobrsyhev et al. 2013; with permission from Wiley) (Bobryshev YV, Shchelkunova TA, Morozov IA, Rubtsov PM, Sobenin IA, Orekhov AN, Smirnov AN. Changes of lysosomes in the earliest stages of the development of atherosclerosis. J Cell Mol Med 2013;17 (5):626–35).

acid reduced the expression of pro-inflammatory genes, such as NF-κB, CCL2, MMP-9, phospholipase 2 and cyclooxygenase 2 in macrophages via the peroxisome proliferator-activated receptor γ (PPARγ) signalling pathway. This resulted in the inhibition of atherosclerosis progression. PUFA can also counterpart the pro-atherosclerotic effects of saturated fatty acids reducing the expression of LOX1 and fatty acid binding protein (Ishiyama et al. 2010). Eicosapentaenoic and dehydroascorbic acid (DHA) have anti-inflammatory properties and can be beneficial in atherosclerosis (Merched et al. 2008). Nitro-fatty acids are formed in oxidative stress conditions as a result of interaction of fatty acids with reactive nitrogen species (Khoo and Freeman 2010). They have been demonstrated to possess anti-inflammatory and atheroprotective properties mediated by Nrf2 and PPARγ signalling (Schopfer et al. 2010). In mouse model of atherosclerosis, treatment with nitro-fatty acids resulted in plaque stabilisation due to increased collagen deposition (Bonacci et al. 2011). While high level of LDL is associated with increased risk of atherosclerosis, HDL is known to have atheroprotective functions, improving cholesterol efflux and metabolism (Rader 2006). These protective effects are partly mediated by the anti-inflammatory activity of HDL. In mouse model of atherosclerosis, normalisation of HDL serum levels resulted in a decrease of pro-inflammatory macrophage numbers in the lesions and to an increase of M2 macrophage markers CD163, Arg-1 and transcription factor FIZZ1 (Feig et al. 2011). The expression of Arg-1 and FIZZ1 was dependent on STAT6 (Sanson et al. 2013). In general, there is a complex interplay between various types of native

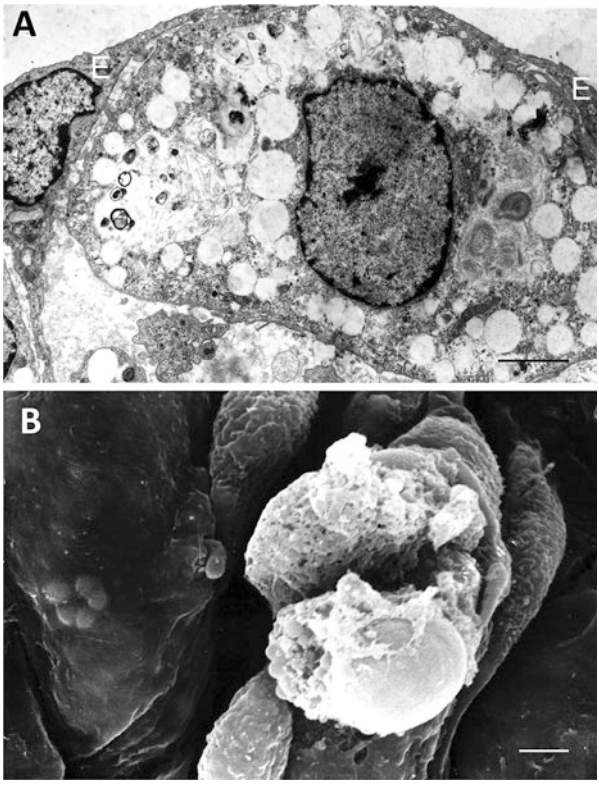

Fig. 11.6 Macrophage foam cells in the intima of the aorta. (**a**) Formation of a foam cell in the subendothelial space in close proximity to the luminal endothelial cells (E). Transmission Electron Microscopy. (**b**) Rupture of the luminal endothelial monolayer accompanied by the exit of a degenerating foam cell to the blood circulation. Scanning Electron Microscopy. Scale bars = 2 μm (**a**, **b**). [Reproduced from Bobryshev 1983 (Bobryshev YV. Morpho-functional characterization of the endothelium of the aorta of rabbits at experimental hypercholesterolemia. Thesis of Candidate of Science. Leningrad, USSR, 1983, 312p)]

and modified lipoprotein particles and macrophage subtypes initiated by them, and more studies are needed to investigate these relationships in detail (Getz and Reardon 2015).

It is well known that the aggregation of foam cells accompanied by the destruction of foam cells in early atherosclerotic lesions eventually leads to the formation of a necrotic core in a growing atherosclerotic lesion (Bobryshev 2006). In advanced atherosclerotic plaques, some macrophage origin foam cells can be formed directly in the subendothelial space in close proximity to the luminal endothelium (Fig. 11.6a). The formation of foam cells in such close proximity to the luminal endothelium can lead to disintegration of luminal endothelial monolayer (Fig. 11.6b) and thus can be considered as one of reasons of plaque rupture (Nagornev et al. 1991).

11.3.4 Macrophage Activation as Therapeutic Target in Atherosclerosis

The prominent role of macrophages phenotypic changes in atherosclerotic lesions indicates the importance of exploring the possibilities of immunocorrective therapy for the treatment of atherosclerotic patients. Monocytes/macrophages isolated from human blood have been used as a model for testing macrophage activation in atherosclerotic patients in comparison with healthy individuals (Orekhov et al. 2015). It has been demonstrated that monocytes/macrophages from atherosclerotic patients had a decreased ability to polarise towards pro- or anti-inflammatory phenotype in response to IFN-γ or IL-4, respectively, as well as a high degree of individual difference in macrophage ability for polarisation between the studied subjects.

Macrophage-based assay has also been successfully used for the evaluation of beneficial effects of potential anti-atherosclerotic substances. Isolated human monocytes/macrophages stimulated with IFN-γ or IL-4 were incubated with extracts of various botanicals: hawthorn flowers (*Crataegus* sp.), elderberry (*Sambucus nigra*), calendula (*Calendula officinalis*), St. John's wort (*Hypericum perforatum*) and violet (*Viola* sp.). Polarisation towards pro- or anti-inflammatory phenotype was assessed by measuring the production of TNF-α and CCL18. It was demonstrated that extracts of hawthorn and St. John's wort caused macrophage depolarisation, reducing the production of both markers, which may be exploited for therapeutic purposes (Orekhov et al. 2015).

The ability of several anti-atherogenic drugs to influence macrophage phenotype was tested on primary monocyte-derived macrophages stimulated or not with IFN-γ for 7 days. Allicor is a garlic powder preparation manufactured by Inat-Pharma (Russia), which possesses the hypocholesterolaemic and anti-atherosclerotic activities (Orekhov et al. 2013). SkQ1 is an antioxidant manufactured by Lomonosov Moscow State University (Russia). Vezugen is a peptide complex (lysine, glutamic acid, aspartic acid) manufactured by JSC Pharm-Sintez (Russia), which improves metabolism in vascular wall cells. Cellex is another polypeptide drug based on pig brain extract manufactured by JSC Pharm-Sintez (Russia), which is aimed to improve cerebral functions. CardioHealth is a plant complex with antihypertensive and moderate hypoglycaemic and hypolipidaemic effects (Ter-Grigoryan et al. 2003; Khavinson et al. 2014; Kulesh and Shestakov 2016). Vezugen, Allikor, Cellex, CardioHealth and SkQ1 (see Appendix) were added to macrophages at the concentrations ranging from 10^{-5} to 10^{-2} μg/ml and the cells were incubated for 24 h. We found that Vezugen, Allikor and CardioHealth had no effect, while Tsellex at concentration 10^{-3} μg/ml caused a decrease of TNF-α expression level in M0 macrophages (Table 11.3). At the same time, SkQ1 at concentration 10^{-4} μg/ml caused a decrease of TNF-α expression level in both of M0 and M1 macrophages.

Macrophage-based model was used for studying anti-atherogenic effects of the previously described experimental drugs with regard to reducing intracellular cholesterol accumulation. Blood sampling was performed prior to the drug administration, as well as in 2 and 4 h after administration. The obtained serum was

Table 11.3 Effect of drugs Vezugen, Allikor, Tsellex, CardioHealth and SkQ1 on the expression of TNF-α by human macrophages[a]

Drug concentration in cell culture (μg/ml)		0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
		Relative expression of TNF-α				
Vezugen n = 2	M0	1 ± 0	0.94 ± 0.02	0.87 ± 0.04	0.91 ± 0.03	0.92 ± 0.02
	M1	1.80 ± 0.14	1.76 ± 0.18	1.77 ± 0.02	1.75 ± 0.17	1.70 ± 0.02
Allicor n = 2	M0	1 ± 0	0.91 ± 0.01	0.83 ± 0.02	0.92 ± 0.01	0.90 ± 0.01
	M1	1.77 ± 0.16	1.79 ± 0.28	1.71 ± 0.20	1.73 ± 0.21	1.75 ± 0.17
Cellex n = 3	M0	1 ± 0	0.95 ± 0.06	0.88 ± 0.06	0.73 ± 0.07[b]	Cytotoxic effect
	M1	1.80 ± 0.09	1.87 ± 0.21	1.84 ± 0.16	1.39 ± 0.27	Cytotoxic effect
CardioHealth n = 3	M0	1 ± 0	0.92 ± 0.04	0.87 ± 0.06	0.93 ± 0.07	0.93 ± 0.01
	M1	1.88 ± 0.23	1.72 ± 0.14	1.79 ± 0.02	1.74 ± 0.10	1.86 ± 0.09
SkQ1 n = 3	M0	1 ± 0	0.77 ± 0.09	0.26 ± 0.06[b]	Cytotoxic effect	Cytotoxic effect
	M1	2.01 ± 0.10	1.68 ± 0.02	0.30 ± 0.01[b]	Cytotoxic effect	Cytotoxic effect

[a]The drugs were added in culture of M0 or M1 macrophages for 24 h at the concentrations from 10^{-5} to 10^{-2} μg/ml. TNF-α gene expression in M0 macrophages was taken as reference
[b]Significant differences from the control (Student's T-test)

evaluated for the ability to induce intracellular cholesterol accumulation in primary cultures of macrophages and was isolated from a healthy donor. Macrophages were incubated with patient serum samples (10%) for 24 h, and intracellular cholesterol concentration was measured. The experimental drugs were demonstrated to reduce cholesterol accumulation in the macrophage-based cellular model (Table 11.4). The description of the experimental drugs is provided in Appendix.

11.4 Conclusion

Macrophages represent a cell type, importantly involved in the development of local inflammatory response and atherosclerotic lesion growth. Macrophages that are present in atherosclerotic lesions belong to either pro-inflammatory phenotype or anti-inflammatory phenotype or alternatively activated phenotype. While pro-inflammatory macrophages promote the inflammatory response and participate in lipid accumulation, anti-inflammatory macrophages are responsible for the inflammation resolution and plaque stabilisation. The growth of atherosclerotic lesions notably depends on the balance between different macrophage phenotype populations. The properties of macrophages relevant to diverse functional

Table 11.4 Anti-atherogenic effects of Allicor, Cellex, CardioHealth and Vezugen[a]

Model:	Ex vivo (A)			In vitro (B)				
	Time after administration			Concentration				
	0 h	2 h	4 h	0 (Control)	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Allicor	100%	67% ± 14	60% ± 12[b]	100%	57% ± 22	50% ± 10	41% ± 14	68% ± 3[c]
Cellex	100%	52% ± 10[b]	34% ± 7[b]	100%	–	74% ± 50	73% ± 24	79% ± 11
CardioHealth	100%	67% ± 42	9% ± 6[b]	100%	15% ± 8[c]	42% ± 13	66% ± 11	69%±2[c]
Vezugen	100%	121% ± 22	84% ± 16	100%	67% ± 34	59% ± 13	70% ± 10	91% ± 22

"–" cytotoxicity

[a]Significant differences from the control (Student's T-test)

[b](A) *Influence of the experimental drug on blood atherogenicity*. Blood sampling was performed at baseline and 2 and 4 h after the drug administration to patients (Table 11.4). The collected serum was evaluated for the ability to induce intracellular cholesterol accumulation in primary cultures of macrophages isolated from a healthy donor. Serum samples (10%) were added to the culture for 24 h, and intracellular cholesterol concentrations were measured. Anti-atherogenic efficacy was assessed by the reduction of cholesterol accumulation in comparison to baseline

[c](B) *In vitro model*. Pooled sera from patients with cardiovascular disease (ischaemic heart disease, hypertension, myocardial disease, heart failure and others) used as an inducer of intracellular accumulation of lipids that caused an increase of intracellular cholesterol by at least 40% relative to healthy donor sera. Culture of primary macrophages was isolated from the blood of a healthy donor and incubated with 30 μl of serum (10%) for 1 day before intracellular cholesterol assessment

predisposition make macrophages to be an attractive therapeutic target in search of novel anti-atherosclerotic treatments.

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Conflict of Interest Disclosure The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix

Information about Allikor, SkQ1, Vezugen, Cellex and CardioHealth

Allicor

Manufacturer: Inat-Pharma (Russia

Composition: 1 tablet contains 300 mg of garlic powder.

Pharmacological effects: hypocholesterolaemic, antiagregatine, fibrinolytic, hypotensive. Reduces cholesterol and triglycerides in the plasma for hyperlipidaemia, slows the development of atherosclerosis, promotes the resorption of existing plaques, reduces blood sugar and blood pressure, inhibits platelet aggregation, normalises the increased blood clotting and promotes lysis of fresh thrombus.

Indications: atherosclerosis, hypertension, myocardial period, diabetes, migraine, impotence, decreased immunity, pregnancy; prevention of myocardial infarction and stroke; postoperative complications in patients with vascular disease, flu and colds.

Contraindications: Hypersensitivity to the drug.

Side effects: None known.

SkQ1

Manufacturer: Lomonosov Moscow State University (Russia)

Composition: SkQ1 is dissolved in 50% aqueous propylene glycol. The three most important segments of the molecule SkQ1 are Plastohinol, a powerful natural antioxidant carrying electrons from the chloroplasts of plants; C10, transports SkQ1 molecule in the cell membrane; Triphenylphosphonium, positively charged group delivering the components in the mitochondria.

Pharmacological effects: SkQ1 blocks and reduces the amount of free radicals formed by cells and thus prevents apoptosis-induced mitochondrial reactive oxygen species.

Indications: SkQ1 part of the eyedrops Vizomitin (Antioxidant, kerato-protektornoe agent for the treatment of early age-related cataract and the syndrome of "dry eye"), as well as part of the MitoVitan serum

Contraindications: Hypersensitivity to the drug.

Side effects: Allergic reactions.

Vezugen

Producer: JSC "pharm" (Russia)

Composition: peptide complex AC-2 (lysine, glutamic acid, aspartic acid). Other ingredients: microcrystalline cellulose, sugar, beet sugar, lactose, starch, Tween-80.

Pharmacological effects: Peptide complex AC-2 has directed tissue-specific effects on the vascular wall. Vezugen promotes normalisation of the functional state of vessels, regulates metabolism in the cells of the vascular wall, improves the condition of the vessel walls and normalises lipid metabolism.

Indications: general and cerebral arteriosclerosis; hypertension; coronary heart disease; endarteritis; of varicose veins of the lower extremities; systemic and local microcirculation disorders; vascular encephalopathy; hypercholesterolaemia; vascular dystonia; psycho-emotional stress; effects of acute stroke; the impact of various factors on the extreme. Vezugen also used for the prevention of vascular disease in the elderly.

Contraindications: individual intolerance to the components of dietary supplements, pregnancy, breastfeeding.

Side effects: None known.

Cellex

Producer: JSC "Pharm-Sintez" (Russia)

Composition in 1 ml: active substance: polypeptides from hog brain of embryos based on 0.9–2.4 mg of total protein (nominal total protein content—1.65 mg per 1 ml of substance); excipients: 3.75 mg of glycine, 0.1 M disodium hydrogen phosphate solution, 5.85 mg of sodium chloride, 0.005 mg of Polysorbate 80, purified water.

Pharmacological effects: The presence of tissue-specific signalling proteins and polypeptides leads to neuroreparation. The drug activates the secondary neuroprotection by stimulating synaptogenesis processes of autophagy recovery signals. Tissue-specific and systemic restorative effect was found as well as the restoration of the regenerative and reparative potential of the brain cells reducing the number of damaged cells and the severity of perifocal oedema in the penumbra, the restoration of microcirculation and perfusion. Recovers and regulates stimulation of different

compartments of central nervous system. The therapeutic effect usually develops within 3–5 days after the start of administration.

Indications: Cerebrovascular diseases.

Contraindications: Epilepsy; Manic psychosis; age of 18 years (due to the lack of clinical data).

Side effects: allergic reactions.

CardioHealth

CardioHealth is a plant complex from the leaves of the European Olive, standardised to oleuropein content (4 mg), Potentilla goose and Andrographis paniculata. CardioHealth has antihypertensive and moderate hypoglycaemic and hypolipidaemic effects.

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Chapter 12
Macrophage Dysfunction in Respiratory Disease

Kylie B.R. Belchamber and Louise E. Donnelly

Abstract In the healthy lung, macrophages maintain homeostasis by clearing inhaled particles, bacteria, and removing apoptotic cells from the local pulmonary environment. However, in respiratory diseases including chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis, macrophages appear to be dysfunctional and may contribute to disease pathogenesis. In COPD, phagocytosis of bacterial species and apoptotic cells by both alveolar macrophages and monocyte-derived macrophages is significantly reduced, leading to colonization of the lung with pathogenic bacteria. COPD macrophages also release high levels of pro-inflammatory cytokines and chemokines, including CXCL8, TGFβ, and CCL2, driving recruitment of other inflammatory cells including neutrophils and monocytes to the lungs and promoting disease progression.

In asthma, defective phagocytosis and efferocytosis have also been reported, and macrophages appear to have altered cell surface receptor expression; however, it is as yet unclear how this contributes to disease progression but may be important in driving Th2-mediated inflammation. In cystic fibrosis, macrophages also display defective phagocytosis, and reduced bacterial killing, which may be driven by the pro-inflammatory environment present in the lungs of these patients.

The mechanisms behind defective macrophage function in lung diseases are not currently understood, but potential mechanisms include alterations in phagocytic receptor expression levels, oxidative stress, but also the possibility that specific diseases are associated with a specific, altered, macrophage phenotype that displays reduced function. Identification of the mechanisms responsible may present novel therapeutic opportunities for treatment.

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12.1 Introduction

As the most abundant leukocyte in the airways, macrophages were, until recently, thought to maintain the sterility of the lung, through phagocytosis of bacteria and by homeostasis via removal of apoptotic cells (Kopf et al. 2015). However, more recent studies have shown that the airways are colonized by a lung microbiome that changes with disease (Han et al. 2012). This suggests that macrophages do not remove all bacteria from the lungs, but nevertheless play an important role in the removal of pathogenic bacteria, inhaled particulates, and dying cells and are key in maintaining pulmonary homeostasis. In chronic lung diseases, this process of phagocytosis appears to become dysfunctional, leading to the presence of specific bacterial populations which may contribute to the underlying pathophysiology of disease (Han et al. 2012). This is further exacerbated by an increase in the number of apoptotic cells in chronic lung disease. Failure of macrophages to clear these cells leads to secondary necrosis, promoting inflammation and contributing to disease pathophysiology (Henson and Tuder 2008). Failure to clear apoptotic cells also promotes maintenance of the macrophage in a pro-inflammatory phenotype that lacks the capacity to resolve inflammation and thus may be key in driving the chronic inflammatory profile observed in many pulmonary conditions.

Studies into the function of pulmonary macrophage in both health and disease have been hampered by availability of cells, particularly from patient populations. Sampling has usually involved bronchoalveolar lavage, which is not without risk to the subject and is not possible in patients with more severe disease. Nevertheless, this technique has provided access to alveolar macrophages. These are not the only macrophage population found in the lung, with other populations identified within the interstitium of the lung that appear to have distinct, functional characteristics that distinguish this population from macrophages found in the airspaces (Frankenberger et al. 2000). Cell surface markers for identification of different macrophage populations have recently been identified (Desch et al. 2016) which will now allow more detailed research into the roles of these populations and their relative contributions to pulmonary disease furthering our understanding of macrophage dysfunction in chronic lung disease that will lead to novel therapeutic opportunities.

12.2 Chronic Lung Diseases

12.2.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is an umbrella term for three underlying pathophysiologies: chronic bronchitis, small airways disease, and emphysema. The contribution of each of these can vary between patients leading to heterogeneity of the population with some patients experiencing one, two, or all

three of the pathophysiologies. Regardless of aetiology, COPD is characterized by a slowly progressive development of airflow limitation that is poorly reversible (Barnes 2004b). Exacerbations of symptoms can occur, leading to increased hospital admissions, morbidity, and mortality (Wedzicha and Donaldson 2003). Respiratory infections are an important feature of exacerbations, with viruses being detected in two-thirds of cases and bacteria being detected in half of cases (Sapey and Stockley 2006). Recent data has shown that bacterial infections often follow viral infection, suggesting a link between an initial viral infection and secondary bacterial infection (George et al. 2014; Mallia et al. 2011). The main bacterial species detected in the COPD lungs are *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*, which are detected despite a 20-fold increase in the number of alveolar macrophages found in the lungs of COPD patients, indicating dysfunctional bacterial clearance in this disease (Hill et al. 2000; Wilkinson et al. 2003). The presence of lower airway bacterial colonization is strongly associated with increased exacerbations and worsening of lung function (Donaldson et al. 2002) leading to more rapid disease progression.

The first studies into phagocytes in COPD utilized the dimorphic fungus *Candida albicans* and showed no difference in phagocytosis by peripheral granulocytes (largely neutrophils) in COPD patients (Ritts et al. 1976); however, later studies indicated reduced phagocytosis of the same species by peripheral monocytes in chronic bronchitis (Nielsen and Bonde 1986). Subsequent observations using alveolar macrophages showed reduced phagocytosis and killing of *C. albicans* in cells from COPD patients compared with controls (Vecchiarelli et al. 1991; Ferrara et al. 1996).

Using more relevant bacterial species, multiple studies have shown defective phagocytosis of *H. influenzae* not only in alveolar macrophages taken from COPD patients compared to healthy controls (Berenson et al. 2006) but also in monocyte-derived macrophages differentiated from peripheral blood from COPD patients and exposed to both *H. influenzae* and *S. pneumoniae* (Taylor et al. 2010). These data support the theory that there exists a systemic macrophage defect in COPD, and that exposure of macrophages in the lungs to toxins and oxidative stress is not the primary cause of this defect, but this pulmonary exposure may impact further upon an already reduced baseline response. Similar experiments using inert latex beads have failed to show a defect in COPD macrophages, indicating that macrophages have the capacity to phagocytose, but that the bacteria-specific response is reduced (Lundborg et al. 2001; Hodge et al. 2003; Taylor et al. 2010).

Alveolar macrophages from COPD patients also display defective efferocytosis (clearance of apoptotic cells), and this is more apparent in current smokers possibly due to modification of extracellular proteins on the macrophage surface due to exposure to cigarette smoke (Hodge et al. 2007; Kirkham et al. 2004); however, the mechanism underlying this has yet to be elucidated but may be due to oxidative stress. Failure to remove apoptotic cells in the lungs, such as airway epithelial cells and T cells, leads to secondary necrosis, release of inflammatory mediators, and promotion of lung damage (Hodge et al. 2005).

The mechanisms behind these defects in the removal of pathogens and apoptotic cells from COPD lungs remain unclear. Phagocytosis of prey depends on recognition and engagement with various receptors expressed on the macrophage cell surface, and it has been postulated that reduced receptor expression might be the reason for impaired clearance of bacteria and apoptotic cells in COPD. Multiple studies have attempted to look into the role of these receptors in COPD macrophages. The mannose receptor (CD206) which recognizes the terminal mannose on bacterial cell wall glycosides has been shown to be reduced in alveolar macrophages from patients with COPD compared with that of nonsmokers (Hodge et al. 2008). The Toll-like receptors (TLR) are also key receptors in the recognition of bacterial proteins. TLR-4, which binds lipopolysaccharide (LPS) found on the cell wall of gram-negative bacteria, has been shown to not be altered in COPD macrophages (Metcalfe et al. 2014); however, TLR2 which binds lipopeptides from *H. influenzae* and *S. pneumoniae* has been shown to be reduced on COPD alveolar macrophages (Droemann et al. 2005). A host of other scavenger receptors are also implicated in macrophage phagocytosis; however, their study has brought about conflicting results. In alveolar macrophages from COPD patients, studies have shown reduced levels of CD31, CD91, CD44, and CD71 (Hodge et al. 2007). However, other studies have not seen differences in expression of CD11b (CR3a), CD14, CD58, CD80, CD7, or human leukocyte antigen-DR (HLA-DR), but have reported reduced expression of CD86 and CD11a (Lofdahl et al. 2006), whereas others have reported no difference in expression of CD44, CD36, CD61, CD14, CD86, or CD40 but did observe reduced expression of HLA-DR and CD80 (Pons et al. 2005).

The role of receptors is further complicated in monocyte-derived macrophages where defects in bacterial phagocytosis were reported, but there were no differences in the number of cell surface recognition molecules between cells from COPD patients and healthy controls (Taylor et al. 2010). As multiple receptors are likely to be involved in the phagocytic process, and different receptors required for the recognition and uptake of different prey, it is likely that changes in a single, specific receptor are unlikely to be responsible for the defects in phagocytosis seen in COPD macrophages. However, downstream signaling pathways may be implicated.

The sphingosine-1-phosphate (S1P) signaling pathways have been shown to be elevated in COPD alveolar macrophages, and antagonism of S1PR5 improved phagocytosis in one study, implicating this pathway in phagocytosis (Barnawi et al. 2015), but further research is required. Other studies have also shown that efferocytosis can be improved by macrolide antibiotics such as azithromycin (Hodge et al. 2008), the antioxidant procysteine (Hodge et al. 2010), or the Nrf2 activator sulforaphane (Harvey et al. 2011) suggesting that this defect can be resolved.

Macrophages have been considered to be the orchestrators of COPD (Barnes 2004a) due to their capacity to produce many of the mediators that are highly expressed in the lungs of these patients (Fig. 12.1). For example, macrophages produce increased levels of CXCL8 (interleukin (IL)-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Russell et al. 2002a; Culpitt

et al. 2003), which drive neutrophil recruitment and macrophage maturation, respectively. They also produce increased levels of CCL2 (Frankenberger et al. 2011), which in turn leads to the recruitment of monocytes into the lung, which subsequently differentiate into macrophages. These cells also produce CXCR3 chemokines, CXCL9, CXCL10, and CXCL11 (Shaykhiev et al. 2009), which could account for the elevated levels of these chemokines in COPD sputum (Costa et al. 2008). Finally, COPD macrophages produce increased levels of proteases including matrix metalloproteinases (MMP) 2 and 9 (Finlay et al. 1997; Russell et al. 2002b), which are considered important in driving destruction of the extracellular matrix associated with emphysema. Furthermore, this inflammatory macrophage phenotype is not amenable to inhibition by the administration of glucocorticosteroids, and as such these cells are considered steroid insensitive (Culpitt et al. 2003). The mechanism underlying this observation has been elucidated and is due to oxidant-mediated downregulation of histone deacetylase (HDAC)2 (Ito et al. 2005) that can be restored by addition of theophylline (Cosio et al. 2004).

These alterations in macrophage function in COPD have led to speculation that there is a specific COPD phenotype or an abnormal skewing of phenotype in this disease. Shaykhiev and colleagues suggested that this skewing was due to cigarette smoking (2009), and, more recently, a distinct macrophage phenotype has been identified in lung tissue that is glucocorticoid insensitive (Chana et al. 2014), suggesting that targeting these aberrant COPD macrophages and either removing them or pushing them toward a "healthy" phenotype could be beneficial in restoring their defective function and halting the progression of COPD.

12.2.2 Asthma

Asthma is defined as reversible airflow obstruction that can be long-term or remitting. It is characterized by persistent airway inflammation, bronchial hyperresponsiveness, and airway remodeling (Busse and Lemanske 2001). While bacterial colonization is not considered a characteristic of stable asthma, patients have a propensity to develop exacerbations, led by viral or bacterial infection as well as other causes such as allergens and occupational exposures (Singh and Busse 2006). The role of macrophages in asthma has been largely overlooked (Park and Christman 2016) as their absolute numbers do not increase compared to healthy subjects (Jeffery 1999); nevertheless, there are clear phenotypic and functional differences in the asthmatic pulmonary macrophage that may contribute to disease pathophysiology (Pappas et al. 2013).

Initial studies looking at macrophage phagocytosis in asthma were performed using opsonized zymosan, a glucan found on the surface of yeast. Sputum macrophages showed a reduction in zymosan uptake in children with eosinophilic asthma (Alexis et al. 2001); however, their studies showed an increase in opsonized zymosan uptake both in vivo and ex vivo in macrophages from mild asthmatics

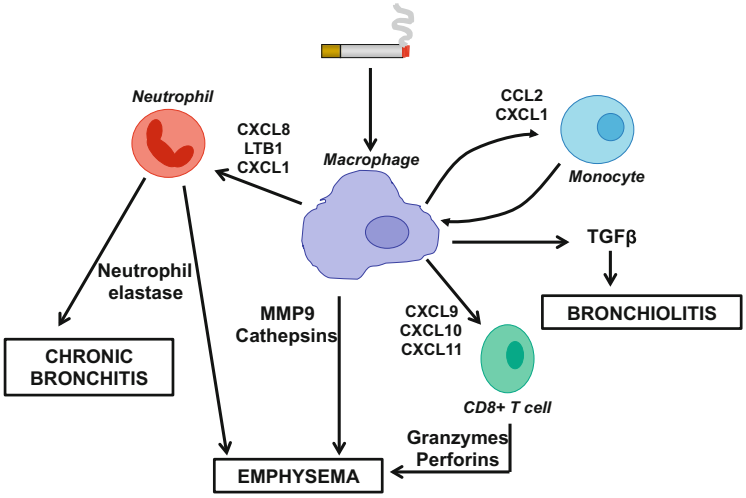

Fig. 12.1 Role of macrophages in COPD pathogenesis. Exposure of the lungs to cigarette smoke causes damage to macrophages and activates pro-inflammatory pathways. Macrophages release an array of cytokines and chemokines to promote monocyte recruitment to the lungs to increase macrophage numbers, recruit neutrophils which release neutrophil elastase, activate CD8+ T cells which release granzymes and perforins, and promote macrophage release of MMP, cathepsins, and TGFβ, all of which contribute to COPD pathogenesis

compared to healthy volunteers (Lay et al. 2009). Further studies showed that in children, there was decreased uptake of *Staphylococcus aureus* in alveolar macrophages from moderate and severe asthmatics compared to healthy controls (Fitzpatrick et al. 2008). Similar data was observed when *H. influenzae* was used as prey in experiments using cells from more severe asthmatics, which was also observed in monocyte-derived macrophages compared to mild asthmatics (Liang et al. 2014). This suggests that, as with COPD, there may be a systemic defect in macrophage phagocytic function and that the local environment is not solely responsible for driving this effect.

Similarly to COPD, efferocytosis has also been shown to be reduced in asthmatic macrophages. Influx of eosinophils to the lungs during asthma leads to increased levels of apoptotic eosinophils in the lungs of asthmatics compared to healthy controls (Walsh 2008; Duncan et al. 2003). Alveolar macrophages from severe asthmatics display reduced uptake of apoptotic cells compared to healthy subjects and those with mild asthma and showed reduced release of cytokines and prostaglandins (Huynh et al. 2005).

Approximately 5% of patients are described as having severe asthma with reduced responsiveness to conventional therapies including glucocorticosteroids (Adcock et al. 2008). Furthermore, similar to COPD, there appears to be a macrophage insensitivity to these drugs (Bhavsar et al. 2008). In addition, there is increased p38 pathway activation (Bhavsar et al. 2008) leading to increased output of a number of inflammatory cytokines. This is accompanied by changes in

phenotype toward an "M2"-like, alternatively activated cell that expresses higher levels of chemokines including CCL17 (Staples et al. 2012) that has been reported to not fully comply with an "M2" phenotype. However, recently Girodet and colleagues have shown that macrophages from severe asthmatic patients show increased levels of CD206 and MHC-II expression together with increases in IL-6, IL-10, and IL-12p40 (Girodet et al. 2016). Moreover, the authors suggest that within this MHC-II^{hi} CD206^{hi} population of macrophages, those that also expressed high levels of histamine receptor (HRH) 1 correlated with increased airway obstruction. They also showed that E-cadherin was highly expressed on these macrophages (Girodet et al. 2016). However, the functional consequence of this finding to macrophage responsiveness is yet to be elucidated.

12.2.3 Cystic Fibrosis

Cystic fibrosis (CF) is a chronic progressive disease characterized by various mutations in the CF transmembrane regulator protein (CFTR) which, in the lung epithelium, leads to reduced uptake of Cl^- ions and reduced hydration of the airway fluid, thereby affecting mucociliary clearance (Chmiel et al. 2002). As with asthma, research into the role of macrophages in this disease has been lacking, but there are clear changes in both the innate immune response and the macrophage in the lung of patients with CF (Bruscia and Bonfield 2016). Exactly how this cell may contribute to disease pathophysiology and progression remains unclear, but there is now increasing interest in this field. For example, the CFTR protein is also expressed by macrophages and is thought to be necessary for the acidification of the phagolysosome required for killing (Di et al. 2006); however, others have failed to see this effect, suggesting that other factors may be important in bacterial killing (Haggie and Verkman 2007). The CF lung is colonized by a number of bacterial species, with *Pseudomonas aeruginosa*, *S. aureus*, *H. influenzae*, and *Burkholderia cepacia* being among the most prevalent (Valenza et al. 2008). Colonization with these bacterial species suggests that there are defects in the innate response that may be attributable to reduced macrophage clearance of these pathogens.

Studies on macrophage phagocytosis in CF have shown varying results, with some studies showing that alveolar macrophages from CF patients display reduced phagocytosis of *Escherichia coli* compared to healthy controls, alongside increased cytokine release (Simonin-Le Jeune et al. 2013), while a study using a more relevant bacteria showed that while phagocytosis of *P. aeruginosa* did not appear to be impaired in CF macrophages, the bacteria had increased survival within the cell (Del Porto et al. 2011). The mechanisms behind any defects in CF macrophages are not clear but may be driven by the lung environment. Bronchoalveolar lavage fluid from CF patients was able to decrease phagocytosis by healthy macrophages, and this has been attributed to the increased levels of neutrophil elastase present (Alexis et al. 2006). Neutrophil elastase cleaves the phosphatidylserine receptor on macrophages, which disrupts efferocytosis indicating a possible mechanism for

reduced macrophage uptake in the CF lung (Vandivier et al. 2002). Toxins released by the bacteria are also able to reduce phagocytosis, including pyocyanin, a toxin released from *P. aeruginosa* which impairs efferocytosis, but not uptake of latex beads, and which could be restored by antioxidants, suggesting an oxidative stress effect on cell surface receptors (Bianchi et al. 2008). Other possible mechanisms include the presence of a thick mucus layer preventing contact between alveolar macrophages and the bacteria themselves, thereby contributing to increased colonization of the lungs (Vandivier et al. 2006).

There is clear evidence that macrophages in the lungs of patients with CF are highly activated, as they display increased release of a number of cytokines (Bonfield et al. 1995) supportive of the concept that these cells contribute to the inflammatory milieu in CF. Further evidence of macrophage dysfunction has arisen from study of sputum macrophages with small sputum macrophages showing decreased expression of the macrophage receptor with collagenous structure (MARCO) and CD206 receptors involved in non-opsonic phagocytosis (Wright et al. 2009). However, further work is required to understand whether these changes in macrophage function are due to the local environment in the CF lung or whether inherent differences in macrophages from these patients are causative.

12.2.4 Other Lung Diseases

As methodology has improved for studies into human pulmonary macrophages together with increased availability of tools to study these cells, there has been increased interest in the role of these cells in other lung disease such as idiopathic pulmonary disease (IPD) and acute respiratory distress syndrome (ARDS). As with other pulmonary conditions, there appears to be a skewing of macrophage phenotype in interstitial lung diseases with an increase in CD40 expression in cells from sarcoid patients and increased CD163 in cells from those with idiopathic pulmonary fibrosis (IPF) (Wojtan et al. 2016). How these observations translate to macrophage function remains to be elucidated, but early studies showed that macrophages from IPF patients were unable to kill bacteria (Savici et al. 1989) with more recent studies showing reduced clearance of apoptotic cells (Morimoto et al. 2012) similar to that seen in COPD. Alveolar macrophages from IPF patients also show increased responses to collagen by releasing increased levels of CCL18, CCL2, and IL-1ra via increased upregulation of CD204 (Stahl et al. 2013), again demonstrating changes in macrophage function which appears characteristic of disease.

12.3 Conclusion

Macrophage dysfunction appears to be common across multiple chronic lung diseases; however, why this is the case remains unclear. There are many proposed mechanisms; however, further work is required in order to elucidate the underlying reasons for these observations, but may relate to differing local microenvironments due to specific pulmonary inflammatory insults, e.g., exposure to cigarette smoke or specific pathogens.

There appears to be common changes in the capacity of macrophages to clear pathogens and/or apoptotic cells, and one theory is that alveolar macrophages are already full or satiated due to clearance of environmental particles (e.g., tar or particles from pollution). The increased presence of bacteria, apoptotic cells, and, in the case of smokers, tar and other particulates could lead to the hypothesis that the cells simply do not have enough capacity to consume any additional material. However, since monocyte-derived macrophages from asthmatics, COPD and CF patients, still show a defective phagocytic phenotype, the concept of satiety cannot solely explain these observations. This instead suggests a systemic defect, which may appear early in the disease process, and, instead of being resultant of the damage in the lungs, actually contributes to drive this process.

As mentioned previously, phagocytosis occurs via a multitude of cell surface receptors and requires complex intracellular signaling mechanisms, actin reorganization, and phagolysosome formation to be successful. It is postulated that any number of these processes could be defective. For example, downregulation of the receptors involved in recognition and phagocytosis of prey would lead to a reduction in the cells' ability to phagocytose. Proteases have been shown to degrade cell surface receptors, and cigarette smoke has been shown to downregulate receptors including CD91, CD31, CD44, and CD71, suggesting oxidative stress may also damage receptors (Hodge et al. 2010), which could be potential mechanisms in both CF and COPD.

Other factors that could also alter macrophage function include viral infection. Viruses are known to be a cause of exacerbations in COPD and asthma, and rhinovirus is able to enter macrophages, although may not be able to replicate inside the cell (Gern et al. 1996). It is possible, therefore, that viruses may alter the function of macrophages during exacerbations in respiratory disease. Oliver et al. demonstrated that this may be the case, showing that rhinovirus suppressed phagocytosis of *E. coli* by alveolar macrophages (2008), and suggesting that treating viral infections may prevent subsequent bacterial infection in respiratory diseases.

Other proposed mechanisms are that inhaled medications could alter cell function; however, there is little evidence to support this theory. In fact, studies have shown that in vitro, budesonide, formoterol, and azithromycin did not alter phagocytosis of *H. influenzae* in monocyte-derived macrophages from COPD patients (Taylor et al. 2010) and that dexamethasone and formoterol had no effect in cells from severe asthmatics (Liang et al. 2014). In vivo, it has been shown that low-dose azithromycin increased efferocytosis of bronchial epithelial cells and neutrophils in

alveolar macrophages from COPD patients, but this was not due to changes in receptor expression (Hodge et al. 2008), indicating that further research is required into the effects of various treatments on improving phagocytosis.

Improving clearance of bacteria by macrophages may prove beneficial in many chronic lung diseases, leading to reduced incidence of exacerbation, and improved morbidity and mortality. Opsonization is known to improve phagocytosis of bacteria (Wellington et al. 2003), and so promoting the production of opsonins in the lungs may improve phagocytosis. However, this could prove difficult in the CF where thick mucus may prevent opsonization from occurring. Increasing receptor expression by macrophages may also increase phagocytosis, demonstrated by the use of sulforaphane, a chemical found in broccoli and other green vegetables, which has been shown to improve phagocytosis of *H. influenzae* by alveolar macrophages via upregulation of the phagocytic receptor MARCO (Harvey et al. 2011).

Other strategies that could be beneficial involve changing macrophage phenotype toward a more homeostatic phenotype and restoring the skewed populations observed in many diseases. Resolvins are lipid mediators that have been described as "specialized pro-resolving mediators" with key roles in the resolution of inflammation including regulating macrophage phenotype by promoting a more anti-inflammatory macrophage and have been shown to reverse the effects of cigarette smoke extract on suppression of phagocytosis (Croasdell et al. 2015). This suggests that manipulation of phenotype might improve bacterial clearance and restore macrophage function to that of a homeostatic cell type and hence be a possible target for therapeutics.

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Part IV
Macrophages as a Target for Biointervention

Chapter 13
Activation of Macrophages in Response to Biomaterials

Jamie E. Rayahin and Richard A. Gemeinhart

Abstract Macrophages are the initial biologic responders to biomaterials. These highly plastic immune sentinels control and modulate responses to materials, foreign or natural. The responses may vary from immune stimulatory to immune suppressive. Several parameters have been identified that influence macrophage response to biomaterials, specifically size, geometry, surface topography, hydrophobicity, surface chemistry, material mechanics, and protein adsorption. In this review, the influence of these parameters is supported with examples of both synthetic and naturally derived materials and illustrates that a combination of these parameters ultimately influences macrophage responses to the biomaterial. Having an understanding of these properties may lead to highly efficient design of biomaterials with desirable biologic response properties.

Abbreviations

Arg	Arginase
BMDM	Bone marrow-derived macrophage
CCR7	C-C chemokine receptor type 7
CD16	Cluster of differentiation 16
CD163	Cluster of differentiation 163
CD200	Cluster of differentiation 200
CD206	Cluster of differentiation 206 or mannose receptor

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CD47	Cluster of differentiation 47
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CFA	Complete Freund's adjuvant
COX-2	Cyclooxygenase-2
CXCL10	C-X-C motif chemokine 10
FBGC	Foreign body giant cell
FGF	Fibroblast growth factor
HLA-DR	Human leukocyte antigen, antigen D related
IC	Immune complex
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-4RA	Interleukin 4 receptor antagonist
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-9	Interleukin 9
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
M(−)	Unstimulated, resting, or naïve macrophage
M(X)	Macrophages stimulated with molecule X
M1	Classically activated
M2	Alternatively activated
M2a	Wound healing macrophage
M2b	Type II macrophage
M2c	Deactivated macrophage
MARCO	Macrophage receptor with collagenous structure
MHCII	Major histocompatibility complex class II
NO	Nitric oxide
p(NIPAm-co-AAc)	Poly(N-isopropyl acrylamide-*co*-acrylic acid)
PBMC	Peripheral blood mononuclear cell
PCL	Poly(ε-caprolactone)
PDMS	Poly(dimethylsiloxane)
PDO	Polydioxanone
PE	Polyethylene
PEG	Poly(ethylene glycol)
PGE_2	Prostaglandin E2 or dinoprostone
PLA	Poly(lactic acid)
PLGA	Poly(lactide-*co*-glycolide)

PMMA	Poly(methyl methacrylate)
poly(HEMA)	Poly(2-hydroxyethyl methacrylate)
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
PVA	Poly(vinyl alcohol)
PVC	Poly(vinyl chloride)
ROS	Reactive oxygen species
TGF-β	Transforming growth factor beta
Th1	Type 1 T helper
Th2	Type 2 T helper
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

13.1 Introduction

Macrophages are the workhorses of the immune system and have powerful and long-lasting impact on the integration or rejection of a biomaterial (Klopfleisch 2016; Garash et al. 2016; Ogle et al. 2016). Macrophages have an ability to influence both tissue integrative and destructive processes, stemming from their relatively plastic nature (Mantovani et al. 2004; Mosser 2003; Gordon 1986; Mosser and Edwards 2008; Gordon and Martinez 2010). In the simplest of conception, macrophages exist in one of two opposing forms: classically activated or alternatively activated phenotypes (Stein et al. 1992; Gordon 1986, 2007; Gordon and Martinez 2010; Gordon and Taylor 2005; Edwards et al. 2006; Mosser and Edwards 2008; Mosser 2003). In reality, macrophages often exist in the spectrum between these extremes (Fig. 13.1). We can understand and characterize macrophages by quantifying their activation-specific characteristics. This complex macrophage phenotype shapes the general reaction to a biomaterial (Mosser and Edwards 2008).

Each macrophage activation state elicits opposing inflammatory and anti-inflammatory reactions in response to the stimulus. One nomenclature for these activation states describes which T-helper cells the macrophages activate. Type 1 T-helper (Th1) cells are activated by M1 macrophages, while type 2 T-helper (Th2) cells are activated by M2 macrophages (Mantovani et al. 2004). Classically activated macrophages promote the inflammatory response and Th1 cells (Mosser and Edwards 2008; Mosser 2003; Gordon 1986). Interferon gamma (IFN-γ) activates macrophages to the classically activated (M1) state, and this phenotype serves in the innate immune response to protect against pathogens or other danger signals in the body, such as fragments of extracellular matrix components (Nathan 2008; Gordon 2007; Dale et al. 2008; Mackaness 1977; O'Shea and Murray 2008; Classen et al. 2009). For this reason, classically activated macrophages are programmed to phagocytize any foreign materials and release mediators that induce cellular death (Mackaness 1977; O'Shea and Murray 2008; Classen et al. 2009; Mosser and

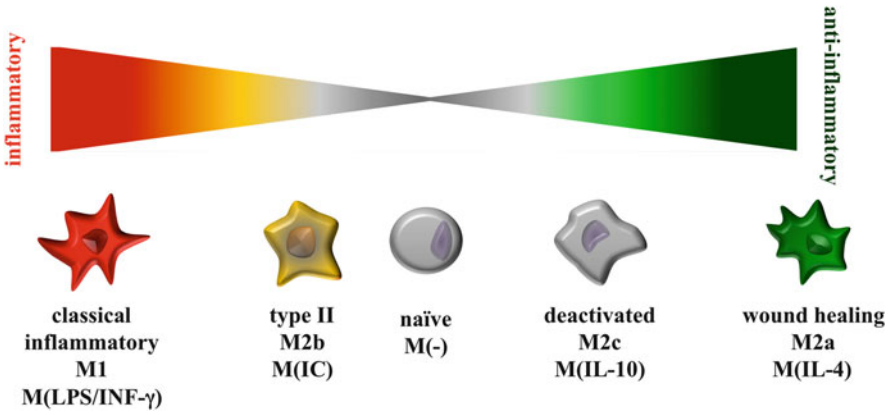
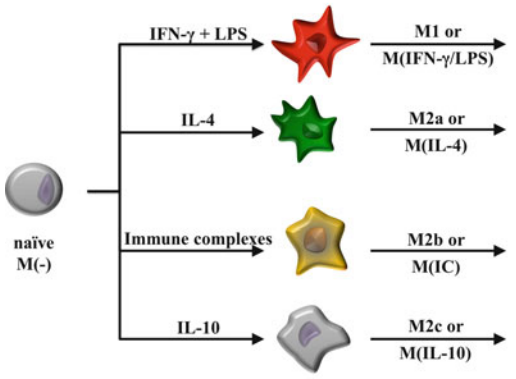


Fig. 13.1 Macrophage activation is a continuum. Macrophages exist in a wide variety of polarization states beyond those characterized in vitro. Classically activated, also known as inflammatory, macrophages and wound healing, also known as anti-inflammatory, macrophages are two extremes of this continuous spectrum. Many stimuli have the ability to polarize macrophages to points within this continuum. Stimuli that yield similar activation, such as LPS, IFN-γ, or the combination of LPS and IFN-γ, have also been identified with all three stimuli resulting in inflammatory macrophages with subtlety different characteristics. Naïve, also referred to as M(−), unstimulated or resting, macrophages lie at the center of the continuum. Type II and deactivated macrophages reside between the inflammatory and anti-inflammatory macrophages where activation characteristics are shared with both inflammatory and anti-inflammatory macrophages. This figure is adapted from several excellent reviews of macrophage activation (Murray et al. 2014; Mantovani et al. 2004; Gordon and Taylor 2005). Abbreviations: *ICs* immune complexes, *IFN-γ* interferon gamma, *IL-4* interleukin 4, *IL-10* interleukin 10, *LPS* lipopolysaccharide, *M1* inflammatory or classically activated macrophage, *M2a* wound healing macrophage, *M2b* type II macrophage, *M2c* deactivated macrophage, *M(−)* unstimulated or naïve macrophages, *M(X)* macrophage stimulated with molecule X

Edwards 2008; Mantovani et al. 2004; Mosser 2003; Gordon 1986; Murray and Wynn 2011). Classically activated macrophages also are potent producers of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 12 (IL-12), and nitric oxide (NO), each signals inflammation to the rest of the body and consequently induces tissue-destructive and foreign body destruction processes (Fig. 13.2).

On the other end of the spectrum is the alternatively activated macrophage, which stimulates Th2 cells (Loke et al. 2007; Edwards et al. 2006; Stein et al. 1992; Mosser and Edwards 2008; Mosser 2003) (Fig. 13.1). Traditionally, this classification has included all macrophage subsets that produce IL-10 and which do not fit the classically activated paradigm. This typically translates to one of three different "alternatively" activated macrophage subsets: wound healing, type II, and deactivated (Mosser and Edwards 2008; Mantovani et al. 2004; Derlindati et al. 2015). While there are a variety of alternatively activated macrophages (M2), literature on these subtypes may refer to any or all of the alternatively activated states as M2 without specifying the subtype. Due to the growing evidence of the



Cytokines	Receptors	Enzymes	Other
$IL\text{-}1\beta_{high}$, $IL\text{-}6_{high}$, $IL\text{-}12_{high}$, $TNF\text{-}\alpha_{high}$, $IL\text{-}10_{low}$	$CD86_{high}$, MHCII, $CD206_{low}$	$iNOS_{high}$, Arg_{low}	NO, ROS
$IL\text{-}10_{high}$, $IL\text{-}1\beta_{low}$, $IL\text{-}6_{low}$, $IL\text{-}12_{low}$, $TNF\text{-}\alpha_{low}$	$CD206_{high}$, MHCII, $CD86_{low}$	Arg_{high} $iNOS_{low}$	polyamines
$IL\text{-}10_{high}$, $IL\text{-}1\beta_{high}$, $TGF\text{-}\beta_{high}$, $IL\text{-}6_{high}$, $TNF\text{-}\alpha_{high}$, $IL\text{-}12_{low}$	$CD86_{high}$, MHCII		NO
$IL\text{-}10_{high}$, $TGF\text{-}\beta_{high}$, IL-4RA $IL\text{-}12_{low}$, $TNF\text{-}\alpha_{low}$, $IL\text{-}1\beta_{low}$, $IL\text{-}6_{low}$	$CD86_{high}$, $CD206_{high}$, $MARCO_{low}$		PGE_2

Fig. 13.2 Macrophage activation is classified by cytokine release, surface receptor expression, enzyme activity, and other reactive molecules. In the presence of IFN-γ and LPS, macrophages become classically activated (M1) and inflammation is stimulated. In the presence of IL-4, alternative activation of macrophages (M2a) occurs allowing the healing of wounds or injury. When stimulated with immune complexes, macrophages are stimulated toward type II activation (M2b) that functions in immunoregulation. If IL-10 is present, deactivated macrophages (M2c) act in immunoregulation and tissue remodeling. For each activation state, other stimulating molecules can be used to achieve a similar activation state. This figure is adapted from several excellent reviews of macrophage activation (Murray et al. 2014; Brancato and Albina 2011; Gordon and Taylor 2005; Biswas and Mantovani 2010; Mantovani et al. 2004). Abbreviations: *Arg* arginase, *CD86* cluster of differentiation 86, *CD206* cluster of differentiation 206 or mannose receptor, *ICs* immune complexes, *IFN-γ* interferon gamma, *IL-1β* interleukin 1β, *IL-4* interleukin 4, *IL-4RA* interleukin 4 receptor antagonist, *IL-6* interleukin 6, *IL-10* interleukin 10, *IL-12* interleukin 12, *iNOS* inducible nitric oxide synthase, *LPS* lipopolysaccharide, *M1* inflammatory or classically activated macrophage, *M2a* wound healing macrophage, *M2b* type II macrophage, *M2c* deactivated macrophage, *M(−)* unstimulated or naïve macrophages, *M(X)* macrophage stimulated with molecule X, *MARCO* macrophage receptor with collagenous structure, *MHCII* major histocompatibility complex class II, *NO* nitric oxide, PGE_2 prostaglandin E2 or dinoprostone, *ROS* reactive oxygen species, *TGF-β* transforming growth factor beta, *TNF-α* tumor necrosis factor alpha, where subscripts low and high indicate low and high expression

wide variety of macrophage polarization profiles, it has been suggested that the nomenclature be changed to classify the subtypes by polarizing stimulus (Murray et al. 2014). Since this review focuses on the response in vivo, typically inflammatory or anti-inflammatory, we will primarily refer to macrophage populations by their activity-based nomenclature: unstimulated (M(−)), inflammatory (M1), wound healing (M2a), type II (M2b), and deactivated (M2c).

Wound healing macrophages (M2a) are induced in vitro by treatment with interleukin 4 (IL-4) or interleukin 13 (IL-13) (Fig. 13.2) (Stein et al. 1992): the original "alternative" activation state. Wound healing macrophages express elevated levels of the non-opsonic mannose receptor (Stein et al. 1992) and fail to

produce nitric oxide through both elevated arginase activity and diminished nitric oxide synthase activity (Mantovani et al. 2004; Edwards et al. 2006; Mosser and Edwards 2008; Mosser 2003; Stein et al. 1992; Gordon 2007). Unlike classically activated macrophages, wound healing macrophages downregulate the production of inflammatory cytokines: TNF-α, IL-1β, IL-6, and IL-12. Additionally, wound healing macrophages upregulate immune-suppressive enzymes, including arginase, and tissue healing cytokines, specifically interleukin 10 (IL-10) (Ferrante and Leibovich 2012; Gordon and Martinez 2010). The wound healing macrophage subset is a potent mediator facilitating and organizing the wound healing process (Stein et al. 1992; Gordon 1986, 2007; Gordon and Martinez 2010; Gordon and Taylor 2005).

Other macrophage subgroups, which have been referred to as alternative macrophages, are type II macrophages (M2b) and deactivated macrophages (M2c) (Fig. 13.2) (Martinez et al. 2008; Mantovani et al. 2004). Both type II and deactivated macrophages share similarities with inflammatory macrophages, but do not directly oppose their actions by producing anti-inflammatory cytokines in the ways that wound healing macrophages do. Therefore, both type II and deactivated macrophages can be thought of as more within the spectrum of activation (Fig. 13.1), somewhere between classically activated and alternatively activated, rather than as two additional extremes of polarization.

Type II (M2b) macrophages are induced by immune complexes and share similarities with inflammatory macrophages (Fig. 13.2). Like wound healing macrophages, type II macrophages produce significant levels of nitric oxide, IL-6, TNF-α, and IL-1β. However, type II macrophages do not produce IL-12. Instead, like wound healing macrophages, they produce IL-10, which results in a Th2 response, as opposed to the Th1 response elicited by inflammatory macrophages (Gordon and Taylor 2005; Gordon 1986; Martinez et al. 2008; Martinez and Gordon 2014; Martinez 2011).

Deactivated macrophages are induced in vitro by IL-10 or glucocorticoids (Fig. 13.2). Like the wound healing macrophages, deactivated macrophages express significant levels of arginase and mannose receptor and do not produce inflammatory mediators. Deactivated macrophages are referred to as "deactivated" because they function in immune suppression and remodeling of the extracellular matrix by expression of factors, such as IL-10 and TGF-β (Martinez et al. 2008; Martinez and Gordon 2014; Gordon and Martinez 2010; Gordon and Taylor 2005).

Macrophages are highly plastic, meaning that macrophage activation is not static and that reprogramming may occur from one polarization state without dedifferentiation or deprogramming. Having biomaterials which promote the desired response, be it pro-inflammatory or anti-inflammatory, is highly desirable to tune overall biologic responses. A wide variety of biomaterial parameters have been shown to promote distinct macrophage responses as discussed below. By understanding these parameters and engineering materials with the appropriate properties, macrophages may be reprogramed to promote desirable responses to biomaterial implants.

13.2 Biomaterial Strategies for the Modulation of Macrophage Phenotype

Biomaterial success is highly dependent on macrophage polarization (Valentin et al. 2009; Greisler et al. 1989; Greisler 1988; Klopfleisch 2016; Garash et al. 2016; Ogle et al. 2016). Being able to predict or define these phenotypes prior to development of the biomaterial may help control therapeutic outcomes (Bryers et al. 2012). On the most simplistic level, one parameter may influence macrophage activation; however, it will be the combined properties that modulate responses to the material. The following sections discuss how selected parameters influence macrophage phenotype. Within each section, a table summarizing the activation of macrophages in response to this parameter is presented.

13.2.1 Size

Macrophage responses to foreign material are size dependent (Table 13.1). Macrophages are master phagocytic cells that scour the body for foreign materials ultimately attempting to phagocytize and destroy the structure. Macrophages can effectively phagocytize materials less than 10 μm in the longest dimension (Fig. 13.3). When material size increases (10–100 μm), single macrophages can no longer phagocytize the structure. Instead, a giant multinucleated cell, called a foreign body giant cell (FBGC), is formed following the fusion of several macrophages in an attempt to phagocytize the material (Anderson et al. 2008). When materials are even larger (bulk implants greater than 100 μm), macrophages undergo fusion and/or frustrated phagocytosis. At this level, it is understood that thickness may also modulate responses, where the thicker the material, the greater the foreign body and fibrotic responses expected (Nichols et al. 2013; Helton et al. 2011; Ward et al. 2002). Frustrated phagocytosis leads to highly inflammatory environment in which macrophages release a significantly elevated quantity of inflammatory cytokines, reactive oxygen and nitrogen species, and proteolytic enzymes in an attempt to degrade the material.

For example, biodegradable particles elicit a largely size-dependent influence on macrophage activation. For instance, poly(lactide-co-glycolide) (PLGA) copolymer microparticles were more inflammatory than their nanoparticle counterparts in J774 murine macrophages (Nicolete et al. 2011). Microparticles with a diameter of 6.5 μm induced the production of significantly more IL-1β and TNF-α from macrophages than nanoparticles with a diameter of 389 nm. PLGA nanoparticles (500 nm) reduced macrophage activation and led to T-cell tolerance and anergy via the scavenger receptor, MARCO, in a mouse model of experimental autoimmune encephalomyelitis, suggesting a role of these nanoparticles in inhibiting inflammatory response and modifying immune activation (Getts et al. 2012).

Table 13.1 Size-dependent influence on biomaterial-induced macrophage activation

Size (μm)	Material	Species	Model	In vitro/in vivo	Macrophage response	Geometry	References
6.5 ± 3.9	PLGA	Mouse	J774	In vitro	M1	Microparticle	Nicolete et al. (2011)
0.389		Mouse	J774	In vitro	M2	Nanoparticle	Nicolete et al. (2011)
0.500		Mouse	C57BL/6	In vivo	M2	Nanoparticle	Getts et al. (2012)
1500–1900	Alginate	Primate	Cynomolgus macaques	In vivo	M2	Implant	Veiseh et al. (2015)
300–1000		Primate	Cynomolgus macaques	In vivo	M1	Implant	Veiseh et al. (2015)

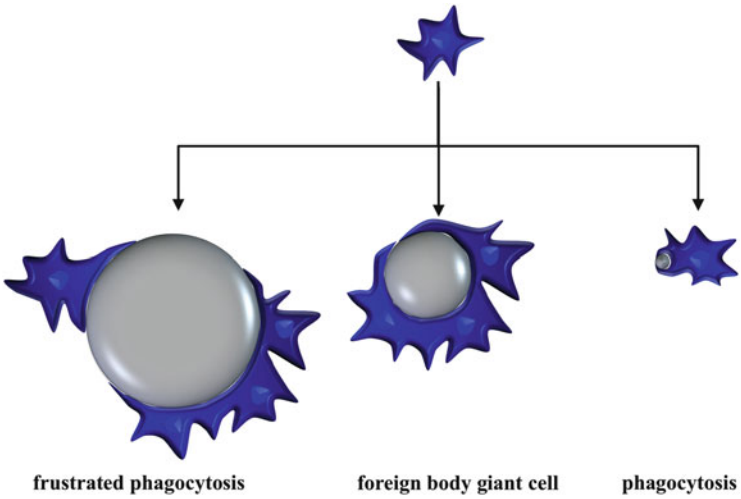


Fig. 13.3 The size of a biomaterial can influence macrophage response. Small particles (<10 μm) can be readily phagocytized, while large particles and materials can result in frustrated phagocytosis and/or foreign body giant cell, a multinuclear phagocyte, formation

Interestingly, the opposite has also been observed although the reasons for the difference can be readily explained. Large spherical implants (1.5–1.9 mm in diameter) were not easily recognized by macrophages, resulting in diminished inflammatory and fibrotic responses when implanted intra-dorsal in nonhuman primates compared to implants which were smaller (0.3–1 mm in diameter) (Veiseh et al. 2015). The diminished inflammation was confirmed to be due to sphere diameter and not total implanted surface area (Veiseh et al. 2015). This illustrated the importance of geometry of the biomaterial, as other geometries induced greater inflammatory response than did spherical implants, suggesting geometry as an additional parameter in understanding immune compatibility.

13.2.2 Geometry

Shape and geometry of the biomaterial have been argued to exhibit the greatest impact on macrophage responses (Table 13.2) (Champion et al. 2007; Champion and Mitragotri 2006). When different geometries, specifically circular, triangular, and pentagonal cross-sectional rods, were implanted in rat gluteal muscles, circular rods produced the smallest foreign body response (Matlaga et al. 1976). Intermediate response was observed for pentagonal rods. Triangular rods elicited the largest foreign body and inflammatory response from surrounding tissue macrophages (Matlaga et al. 1976). The angular aspect determined the severity of the foreign body response, suggesting that smooth, well-contoured implants have reduced inflammatory response than do those with acute angles (Salthouse 1984). More

Table 13.2 Geometry-dependent effects on biomaterial-induced macrophage activation

Shape	Material	Species	Model	In vitro/ in vivo	Macrophage response[a]	Architecture	References
Disks	PDO	Mouse	C57BL/6 BMDM	In vitro	M2	Electrospun fiber	Garg et al. (2009)
Disks		Human	PBMC	In vitro	M2	Electrospun fiber	Garg et al. (2013)
Circular rod	PVC, PE, PU, silicone, Teflon	Rat	Long-Evans	In vivo	M2	Implant	Matlaga et al. (1976)
Pentagonal rod		Rat	Long-Evans	In vivo	M1/M2	Implant	Matlaga et al. (1976)
Triangular rod		Rat	Long-Evans	In vivo	M1	Implant	Matlaga et al. (1976)
Ellipsoid	PS, PVA	Rat	NR8383 alveolar	In vitro	M2	Particles	Champion and Mitragotri (2006)
	PS	Mouse	RAW 264.7	In vitro	M2	Prolate	Sharma et al. (2010)
	PS	Mouse	RAW 264.7	In vitro	M1	Oblate	Sharma et al. (2010)
Spherical	PS, PVA	Rat	NR8383 alveolar	In vitro	M1	Particles	Champion and Mitragotri (2006)

[a]M1/M2 indicates mixed phenotype observed

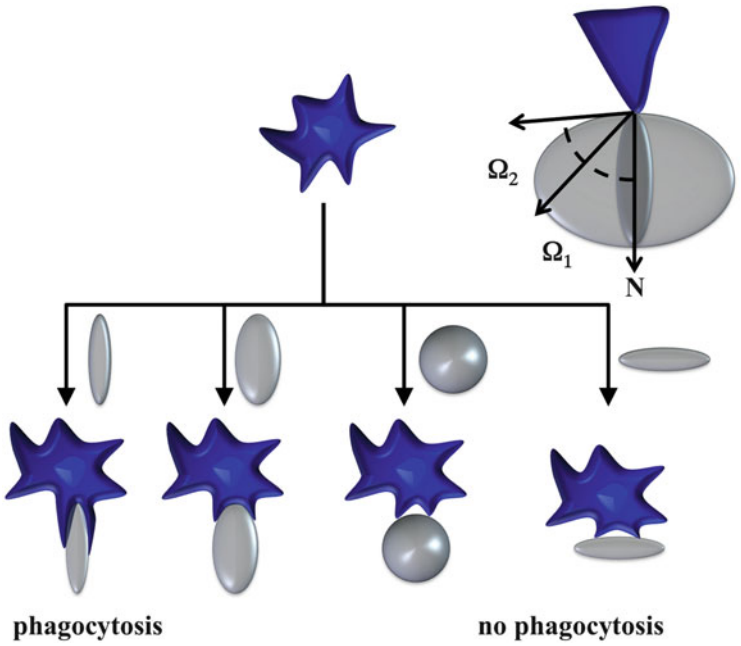


Fig. 13.4 Phagocytic response of macrophages to particles with varying curvature. When the curvature, or angle between the edge of the object at point of contact and a line normal (N) to the point of contact represented by Ω, is small (<45°), the macrophage will attempt to phagocytize the particle as represented by Ω_1. When the curvature is large as represented by Ω_2, macrophages are unable to phagocytize the particle. This figure is, in part, adapted from Moon et al. (2012)

recently, the angle of curvature at the point of macrophage attachment to the biomaterial was found to influence phagocytic activity and the rate at which it is undertaken (Fig. 13.4). Macrophages exposed to material surfaces with curvature greater than 45° had significantly less phagocytic activity (Champion and Mitragotri 2006).

Well-contoured shapes, such as disks, also resulted in alternatively activated and anti-inflammatory activities. Human macrophages, derived from peripheral blood mononuclear cells (PBMCs) and cultured on polydioxanone (PDO) disks, produced enhanced transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) over a 28-day period compared to macrophages cultured on tissue culture polystyrene (Garg et al. 2009). Oblate ellipsoid disks made of polystyrene were taken up to a greater extent than prolate ellipsoids, suggesting that macrophages, which attached to prolate ellipsoids, were deactivated (Sharma et al. 2010). Anti-inflammatory and deactivated macrophage responses to biomaterials may be due to the aspect ratio of the material and the degree of actin remodeling required for the macrophage to phagocytize the material. In general, curved biomaterials with larger aspect ratios require more actin remodeling for uptake and are considered macrophage deactivating due to their

inability to induce phagocytosis (Gratton et al. 2008; Sharma et al. 2010; Champion et al. 2007; Champion and Mitragotri 2006). Despite these studies, consensus on a well-defined immune-compatible shape of an implant has not been defined and continues to be debated (Helton et al. 2011). Further complicating the general geometric recognition by macrophages is the larger architectural presentation of the material.

13.2.3 Architecture

In addition to the size of an implanted biomaterial, the architecture of the material may also influence macrophage response: the roughness of a surface; three-dimensional arrangement of two-dimensional structures, such as gratings or fibers; or porosity (Table 13.3). Early studies have shown that macrophages prefer to adhere to rougher surfaces (Rich and Harris 1981), but roughness also influences their activation status. When murine macrophages (P388D1) were cultured on poly (methyl methacrylate) (PMMA), surfaces with grooves 10 μm wide and 0.5 μm deep, macrophages were elongated and had enhanced migratory capacity (Wojciak-Stothard et al. 1996). Additionally, microgrooves on polystyrene beads increased the number of phagocytic macrophages, with groove depth being the predominant factor influencing migration and phagocytosis of the macrophages (Rich and Harris 1981). Larger grooves on surfaces enhanced classical activation of macrophages. Differences in surface topographies on titanium also affected the inflammatory cytokine profile of murine macrophages when introduced in the presence of lipo-polysaccharide (LPS) (Refai et al. 2004). Rougher surfaces synergistically induced elevated IL-1β, IL-6, and TNF-α mRNA transcription following LPS exposure compared to smoother surfaces, suggesting that these surfaces prompted classical activation in macrophages.

Other patterned architectures such as gratings have been used to regulate cellular interactions through a phenomenon called contact guidance (Dvir et al. 2011). On substrates made of poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), and poly (dimethylsiloxane) (PDMS), parallel gratings influenced macrophage activity. RAW 264.7 cells produced lower levels of TNF-α and VEGF as surface grating size increased, where macrophages exposed to 1 μm diameter gratings produced the lowest levels of both cytokines (Chen et al. 2010). In vivo, macrophage fusion into foreign body giant cells was reduced with grating size of 2 μm compared to planar controls or 500 μm gratings. The changes were topography induced and determined to be independent of material chemistries.

Fibrous architectures, such as electrospun polymer meshes, have patterns which most closely resemble native microenvironments and that macrophages should respond similarly to native matrix (Wang et al. 2013). For instance, bone marrow-derived macrophages grown within electrospun PDO fibers enhanced characteristics of alternatively activated macrophages, specifically increasing expression of arginase compared to inducible nitric oxide synthase (iNOS) along

Table 13.3 Architecturally mediated effects on biomaterial-induced macrophage activation

Architecture	Material	Species	Model	In vitro/ in vivo	Macrophage response[a]	Material type	References
Grooved	PMMA	Mouse	P388D1	In vitro	M2	Substrata	Wojciak-Stothard et al. (1996)
Grooved	PS	Mouse	Peritoneal macrophages from Swiss white mice	In vitro	M1	Beads	Rich and Harris (1981)
Rough/coarse	Titanium	Mouse	RAW 264.7	In vitro	M1	Disk	Rich and Harris (1981)
Small parallel grating	PCL, PLA, PDMS	Mouse, rat	RAW 264.7, Sprague-Dawley	In vitro/ in vivo	M1	Film	Chen et al. (2010)
Large parallel grating		Mouse, rat	RAW 264.7, Sprague-Dawley	In vitro/ in vivo	M2	Film	Chen et al. (2010)
Mesh	PDO	Mouse	BMDM	In vitro	M2	Electrospun	Garg et al. (2013)
Fibrous mesh	PLLA	Mouse	RAW 264.7	In vitro	M2	Electrospun	Saino et al. (2011)
Flat mesh	PLLA	Mouse	RAW 264.7	In vitro	M1	Electrospun	Saino et al. (2011)
Small fibers	PP, PE, PLA, PU	Rat	Sprague-Dawley	In vivo	Less inflammatory	Electrospun	Sanders et al. (2002); Sanders et al. (2000)
Large fibers	PP, PE, PLA, PU	Rat	Sprague-Dawley	In vivo	M1	Electrospun	Sanders et al. (2002); Sanders et al. (2000)
Nonporous	PU	Human	PBMC (CRL-9855)	In vitro	M1	2D films	McBane et al. (2011)
	Poly (HEMA)	Mouse	*BAT-gal*	In vivo	M1	Spherical implant	Sussman et al. (2014)
Porous	Poly (HEMA)	Human	*PBMC (CRL-9855)*	In vitro	M1/M2	3D porous scaffold	McBane et al. (2011)
		Rat	*Sprague-Dawley*	In vivo	M1/M2	Disks	Palmer et al. (2014)
		Human	–	In vivo	M2	Disks	Fukano et al. (2006)
		Mouse	*BAT-gal*	In vivo	M2	Spherical implant	Sussman et al. (2014)

[a]M1/M2 indicates mixed phenotype observed

with TGF-β, VEGF, and FGF (Garg et al. 2013). However, fiber diameter and alignment influenced macrophage activation. Aligned electrospun poly(L-lactic acid) (PLLA) fibers with micro- or nanoscale diameter enhanced macrophage adhesion when compared to randomly aligned or flat PLLA (Saino et al. 2011). In the presence of LPS, macrophages grown on fibrous PLLA secreted significantly less inflammatory cytokines than those cultured on flat PLLA films, suggesting that fibrous materials reduce classical activation in macrophages. In an in vivo multi-material study, fibers (1–5 μm diameter), regardless of material, led to smaller fibrous capsule formation than larger fibers (11–15 μm diameter) (Sanders et al. 2000, 2002).

Internal architecture of the material, such as porosity, may also interplay with other material properties to influence macrophage response. Highly porous materials, or those with defined size of pores or high total porosity, have been shown to have higher ratios of macrophage infiltration (Mitragotri and Lahann 2009). The presence of pores has also been shown to modulate macrophage responses and biointegration (Winter 1974; Sussman et al. 2014; Anderson and Miller 1984). For instance, human monocyte-derived macrophages enhanced TNF-α release when cultured on two-dimensional nonporous films made up of degradable, polar, hydrophobic, ionic polyurethane (PU), over a 28-day period, with the most significant TNF-α release at day 7. Although release of TNF-α waned over time, IL-10 release did not change significantly, suggesting that these macrophages remained in the classically activated state and did not transition to alternatively activated macrophages in response to the nonporous polymer films (McBane et al. 2011). Porous polyurethane scaffold, however, tended to induce enhanced migratory capacity of macrophages and reduced secretion of inflammatory cytokines compared to the films, suggesting an importance in porosity in modulating response to the material (McBane et al. 2011). In a human wound model, porous poly(2-hydroxyethyl methacrylate) (poly(HEMA)) disks showed enhanced healing and biointegration within the wound space (Fukano et al. 2006). When porous poly(HEMA) rods were implanted into the dorsal skin of C57BL/6 mice, rods showed signs of integration with the surrounding tissue (Isenhath et al. 2007). Both of these are examples of wound healing responses largely mediated by the wound healing macrophage (Gordon 2007; Sunderkotter et al. 1994; DiPietro 1995; Koh and DiPietro 2011; Mosser and Edwards 2008; Gordon and Martinez 2010), suggesting that porous scaffolds recruit or promote alternative macrophage polarization.

The size of pores is also important to modifying macrophage phenotype. Hydrogels composed of poly(HEMA) with 34 μm pores and 160 μm pores as well as nonporous hydrogels elicited differing macrophage activation responses (Sussman et al. 2014). When hydrogels were implanted, macrophages responded with significant fibrotic foreign body response in nonporous implants compared to porous ones, suggesting that macrophages took on a classically activated phenotype in response to the implant without pores. While the porous scaffolds had fibrotic responses as well, the implant with the smallest pore size (34 μm) elicited the least fibrotic response. Interestingly, histological analysis of macrophages directly surrounding the pores in the porous scaffolds showed that macrophages attached to and

directly surrounding the 34 or 160 μm pores actually enhanced the expression of classically activated markers, IL-1 receptor and iNOS, compared to those nonadherent to the pores, which showed enhanced mannose receptor expression (greatest in implants with 34 μm pores). While responses to a material may be based off of specific architectural cues, a fine interplay between internal and overall architectural cues determines totality of macrophage response.

Therefore, the response to porous scaffolds is not always easily predictable. For instance, polyurethane porous foams, 7 mm in diameter and 3 mm thick, were surrounded by tissue macrophages 6 weeks after implantation regardless of porosity. Polyurethane foams were positive for macrophages expressing CD80, CD68, mannose receptor, and iNOS (Table 13.3) (Palmer et al. 2014). Additionally, the foams were positive for foreign body giant cells expressing high levels of CD80 and absent levels of CD163, suggesting that these materials promoted a classically activated macrophage phenotype. Expression of mannose receptor, however, suggested a mixed phenotypic profile in response to the porous polyurethanes. An absence of CD80-positive and iNOS-positive cells was noted unless they were in direct contact with the material, suggesting that expression of these markers was dependent on direct activation by the underlying biomaterial. Once again, this suggests that it is the combination of the physicochemical properties of the biomaterial interplay to induce macrophage activation of either phenotype. There is a finely tuned response to architectural cues, and small changes may lead to significantly different macrophage responses.

13.2.4 Hydrophobicity

Hydrophobicity (Table 13.4) has long been regarded as a simple predictor of macrophage response. Under most healthy and normal biologic conditions, hydrophobic biologic surfaces are buried from the external environment and only become exposed during disease-related processes (Seong and Matzinger 2004; Matzinger 1994). Therefore, hydrophobicity is regarded as a danger-associated molecular pattern, and macrophages may respond to it as foreign and necessary to remove and destroy. As such, it is unsurprising that hydrophobicity of biomaterials has been shown to correlate with an increase in the number of inflammatory or classically activated macrophages (Seong and Matzinger 2004; Rostam et al. 2016; Bygd et al. 2015; Akilbekova et al. 2015). The increase in recruitment and local activation of classically activated macrophage subtypes in response to these materials can be explained as largely due to the nature of the hydrophobic surfaces and protein adsorption (Vroman 1962). For instance, nonpolar surfaces facilitated protein unfolding through decreased unfolding free energy barriers (Anand et al. 2010; Vroman 1962). The result of protein unfolding and refolding is a highly disorganized layer of proteins with newly exposed conformations, sometimes referred to as cryptic sites, on the surfaces. These cryptic sites generally lead to inflammatory

Table 13.4 Hydrophobicity of biomaterial and macrophage activation

Hydrophobicity	Material	Species	Model	In vitro/ in vivo	Macrophage response	Material type	References
Hydrophobic	Polystyrene	Human	PBMC	In vitro	M1	Polystyrene disk	Rostam et al. (2016)
	Ether and phosphonic acid-modified p (NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M1	Nanoparticles	Bygd et al. (2015)
	Latex	Mouse	RAW 264.7	In vitro	M1	Beads	Akilbekova et al. (2015)
	Silicone	Human	–	In vivo	M1	Implant	Barker et al. (1978)
	Silicone	Human	PBMC	In vitro	M1 to M1	Silicone foil	Vijaya Bhaskar et al. (2015)
	Silicone	Human	PBMC	In vitro	M2 to M1	Silicone foil	Vijaya Bhaskar et al. (2015)
	Silicone	Human	PBMC	In vitro	cytokine naive to M1	Silicone foil	Vijaya Bhaskar et al. (2015)
Hydrophilic	Poly(acrylic acid)	Rat	Sprague-Dawley	In vivo	M2	Implant	Brodbeck et al. (2002)
	Collagen/hyaluronan	Human	PBMC	In vitro	M2	Implant	Kajahn et al. (2012)
	Collagen/hyaluronan	Human	PBMC	In vitro	M1 to M2	Implant	Franz et al. (2013)
	Hyaluronan/gelatin	Human	PBMC	In vitro	M2	Carbylan-GSX	Hanson et al. (2011)
	Hyaluronan	Human	–	In vivo	M1	Cross-linked dermal filler	Edwards and Fantasia (2007)
	Hyaluronan	Human	–	In vivo	M1	Cross-linked dermal filler	Rongioletti et al. (2003)
	Hyaluronan	Human	–	In vivo	M1	Cross-linked dermal filler	Wolfram et al. (2006)
	Hyaluronan	Human	–	In vivo	M1	Cross-linked dermal filler	Vargas-Machuca et al. (2006)
	Hyaluronan	Human	–	In vivo	M1	Cross-linked dermal filler	Klein (2004)
	Hyaluronan	Human	–	In vivo	M1	Cross-linked dermal filler	Fernandez-Acenero et al. (2003)
	Dextran	Mouse	BALB/c	In vivo	M1	Sephadex (cross-linked dextran)	Blanckmeister and Sussdorf (1985)

responses from macrophages consistent with a classically activated phenotype (Seong and Matzinger 2004).

Natural responses to hydrophobic materials, such as silicone rubbers, have shown questionable histocompatibility with an increase in chronic inflammation with accumulation of monocytes and lymphocytes in the areas surrounding the implant (Iribarren et al. 2002; Barker et al. 1978). Human PBMC-derived macrophages show inflamed activation in response to silicone after being cultured on it for 24 h (Vijaya Bhaskar et al. 2015). Nonactivated human PBMC-derived macrophages showed higher levels of IL-8, C-X-C motif chemokine 10 (CXCL10), and IFN-γ when cultured on silicone compared to tissue culture plastic suggesting not only activation but reprogramming of the macrophages. Alternatively activated human PBMC-derived macrophages also secreted higher levels of TNF-α, interleukin 17 (IL-17), IFN-γ, IL-6, interleukin 9 (IL-9), and CXCL10 when cultured on silicone compared to tissue culture plastic. Finally, classically activated human PBMC-derived macrophages cultured on silicone exhibited higher levels of IL-8 and IFN-γ compared to those cultured on tissue culture plastic. Therefore, macrophages cultured on silicone, regardless of initial macrophage activation state, became more classically activated compared to those grown on tissue culture plastic, suggesting that silicone had the ability to classically activate human PBMC-derived macrophages.

Hydrophilic materials have generally shown the opposite effects. Hydrophilic anionic surfaces promoted anti-inflammatory IL-10 expression, reduced IL-8 expression, and inhibited macrophage fusion into foreign body giant cells, which indicated that these surfaces promoted an anti-inflammatory or alternatively activated macrophage response (Brodbeck et al. 2002). This may be due to the fact that on wettable surfaces, proteins are adsorbed in lower concentrations and in a more native state. In addition, protein reorganization on the surface provides binding sites for cell adhesion and not just cryptic site recognition (Anand et al. 2010). Depending upon the hydrophilic nature, however, proteins are bound with weaker forces which leads to protein detachment and poor or disabled cellular attachment. Hydrophilic surfaces also promote apoptosis of macrophages, through an unknown mechanism, thereby inhibiting their ability to form foreign body giant cells. The combination of which may further explain the lack of an inflammatory response to these types of materials (Brodbeck et al. 2001, 2002).

The macrophage response to hydrophobicity of a material is not always predictable as hydrophilic materials may also induce classical macrophage activation. For instance, cross-linked hydrophilic dextran induced classical macrophage activation (Blanckmeister and Sussdorf 1985). BALB/c peritoneal macrophages took on a polarization status not significantly different from that of macrophages treated with complete Freund's adjuvant (CFA). In other words, these macrophages were classically activated and had antitumor activity both in vitro and in vivo. Sometimes the same material may elicit widely different macrophage responses in different contexts. For instance, when hydrophilic hyaluronan was cross-linked with gelatin in an implant to support human mesenchymal stem cells, human macrophages responded with characteristics of alternatively activated macrophages, specifically

expressing low levels of cluster of differentiation (CD16), high levels of cluster of differentiation 206 (CD206), and low levels of HLA-DR expression after culture for 7 days (Hanson et al. 2011). However, cross-linked injectable hydrophilic hyaluronan implants have also been shown to elicit inflammatory or granulomatous reactions in humans over time (Edwards and Fantasia 2007; Rongioletti et al. 2003; Wolfram et al. 2006; Vargas-Machuca et al. 2006; Klein 2004; Fernandez-Acenero et al. 2003). Confounding these findings is the ability of hyaluronan to degrade into smaller fragments over time, making it unclear if the histological evidence of classical activation is due to the native material or the result of degradation of hyaluronan into smaller fragments.

In some instances, increasing hydrophobicity of a biomaterial, i.e., those with a water-air contact angle between 80° and 90°, leads to anti-inflammatory responses consistent with an alternative macrophage activation (Rostam et al. 2016). Possibly explaining these apparent contradictions, water contact angles can be subdivided into hydrophilic regimes, specifically contact angles between 0° and 45° representing "conditionally" nonadhesive regime, between 45° and 75° representing adhesive hydrophilic regime, and above 75° representing a nonadhesive regime. Materials in either of the nonadhesive regimes had the greatest potential for stimulating wound healing macrophage activation (Richards et al. 2012).

Another explanation for the differences in activation with hydrophilic materials resides in the charge of the surface. Hydrophilic neutral and anionic materials stimulated classically activated macrophages inducing production of IL-8, IL-1β, IL-6, and TNF-α, with low levels of IL-10 (Chang et al. 2008). On the other hand, hydrophilic cationic material surfaces promoted alternative macrophages activation (Chang et al. 2008). This suggests that while hydrophobicity of a material may have a role in mediating a certain response, it is not the sole material property that controls this.

13.2.5 Surface Chemistry

While the size, geometry, hydrophobicity, and surface topography of the surface have been shown to mediate macrophage responses, the surface chemistry (Table 13.5) of the material also dictates how much and what kind of proteins will adsorb onto the surface of the biomaterial and also plays a major role in determining macrophage responses to biomaterials. To examine the influence of varying surface chemistries, self-assembled monolayers were prepared on gold-yielding surfaces with similar physical properties (Barbosa et al. 2006). An increasing number of classically activated macrophages were recruited to and collected from the inflammatory exudates of implants functionalized with a methyl ($-CH_3$) compared to no functionalization. Surface functionalization with hydroxy ($-OH$) and carboxylic acid ($-COOH$) groups elicited intermediate numbers of macrophages. Surfaces coated with the methyl groups induced an in vivo capsule

formation, which was the largest out of all the functionalized groups, suggesting that the methyl-modified surfaces promoted the most significant inflammatory response from the macrophages. While hydroxy-modified surfaces recruited high levels of inflammatory macrophages, these surfaces did not induce the formation of a fibrous capsule to the extent of the methyl-coated surfaces, suggesting that the response elicited by this surface chemistry was not as polarizing as the methyl-modified surfaces. Unfortunately, the hydrophobicity could not be decoupled from the surface chemistry in this case.

Similarly, macrophage interaction with the differing surface chemistries was examined by formulating micron-sized particles made of polypropylene surface-modified with either hydroxy, amine, fluorocarbon, or carboxylic acid groups (Kamath et al. 2008). Using microparticles, macrophages had the opportunity to interact with the surface functionalities and potentially phagocytize the particles. Surfaces with hydroxy and amine surfaces induced thick fibrous capsule formation and cellular infiltration characteristic of inflammation in response to the implant. On the other hand, microparticles with surfaces of carboxylic acid or fluorocarbon modifications had the lowest inflammatory and fibrotic responses.

More recently, microparticles functionalized with acetal, ketone, and nitro groups on the surface induced macrophage infiltration into the implant site, with a lower ratio of classically activated to alternatively activated macrophages present in the site (Bygd et al. 2015). Macrophages exposed to these surfaces produced low levels of TNF-α, high levels of IL-10, and high levels of arginase compared to iNOS, suggesting that functionalization with those groups provoked a more alternatively activated state of the macrophage. On the other hand, sulfone, sulfonic acid, alkene, and epoxide groups on the surface did not stimulate significant macrophage infiltration into implant site, but these materials elicited a higher presence of classically activated macrophages. These macrophages tended to upregulate TNF-α production while decreasing IL-10 and arginase. Some functional groups analyzed yielded macrophage phenotypes which lay within the spectrum, not clearly classically activated or alternatively activated. For instance, particles functionalized with ester, ketal, acetal, sulfone, and oxime expressed intermediate levels of all cytokines or had mixed cytokine profiles, suggesting that either intermediate activation took place or that populations of macrophages polarized both alternatively and classically.

Modification of material with polymers also yields variable surface chemistry characteristics. For instance, mouse macrophages exposed to chitosan scaffolds exhibited properties dependent on the degree of acetylation of the chitosan. When implanted in mice, macrophages surrounding chitosan scaffolds with 5% acetylation were alternatively activated compared to those surrounding 15% acetylated chitosan, which were classically activated, as detected from immunofluorescent staining for CD206 and CCR7 (Vasconcelos et al. 2013). Additionally, there was a reduced IL-6 and TNF-α cytokine secretion of the 5% acetylated chitosan scaffolds compared to 15%, and the 5% acetylated scaffolds elicited enhanced secretion of anti-inflammatory IL-4 compared to 15% acetylation. Similarly, sulfonation of polyurethanes influences macrophage activation (Hunt et al. 1996). Both sulfated

Table 13.5 Influence of surface chemistry on macrophage activation

Surface chemistry	Material	Species	Model	In vitro/ in vivo	Macrophage response[a]	Material type	References
Methyl	Gold	Mouse	BALB/c	In vivo	M1	Monolayers over gold	Barbosa et al. (2006)
Hydroxy	Gold	Mouse	BALB/c	In vivo	M1/M2	Monolayers over gold	Barbosa et al. (2006)
	Polypropylene	Mouse	BALB/c	In vivo	M1	Microparticles	Kamath et al. (2008)
Carboxylic acid	Gold	Mouse	BALB/c	In vivo	M1/M2	Monolayers over gold	Barbosa et al. (2006)
	Polypropylene	Mouse	BALB/c	In vivo	M2	Microparticles	Kamath et al. (2008)
Amine	Polypropylene	Mouse	BALB/c	In vivo	M1	Microparticles	Kamath et al. (2008)
Fluorocarbon	Polypropylene	Mouse	BALB/c	In vivo	M2	Microparticles	Kamath et al. (2008)
Acetal	p(NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M2	Nanoparticle	Bygd et al. (2015)
	Chitosan	Mouse	BALB/c mice	In vivo	M1 or M2 dependent on acetylation	Implant	Vasconcelos et al. (2013)
Ketone	p(NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M2	Nanoparticle	Bygd et al. (2015)
Nitro	p(NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M2	Nanoparticle	Bygd et al. (2015)
Sulfone	p(NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M1	Nanoparticle	Bygd et al. (2015)
Sulfonic acid	p(NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M1	Nanoparticle	Bygd et al. (2015)
Alkene	p(NIPAm-co-AAc)	Mouse	SKH1-E	In vivo	M1	Nanoparticle	Bygd et al. (2015)
Sulfate	Polyurethane	Rat	Lister rat, Liverpool strain	In vivo	M1	Square sheet	Hunt et al. (1996)

[a]M1/M2 indicates mixed phenotype observed

and unsulfated PUs had similar amounts of TNF-α mRNA expression and release around the site of implantation in rats over a 14-day period. Although some specific surface chemistries and modifications have been found to elicit clear activation of macrophages, further study of the specific activation states and the reasons for these activations are needed.

13.2.6 Substrate Mechanics

Substrate stiffness and applied forces on substrates also influence macrophage activation (Table 13.6). In disease, tissue and extracellular matrix stiffness has been shown to change (Cox and Erler 2011; Bidan et al. 2015; Werfel et al. 2013); however, the relationship with macrophage phenotype in vivo has yet to be clearly elucidated. In vitro, clear relationships between substrate mechanics and macrophage activation are emerging.

In the absence of stimulation by LPS, murine macrophages (RAW 264.7) showed no differences in the cytokine expression on poly(ethylene glycol) hydrogels with stiffness ranging from 130 to 840 kPa (Blakney et al. 2012). However, in the presence of LPS, expression of TNF-α, IL-10, IL-1β, and IL-6 increased as implant rigidity increased. As such, stiffer implants tended to induce a more severe foreign body reaction than softer implants. These results were confirmed in vivo with increasing hydrogel stiffness correlating with a more severe foreign body response and activated macrophages surrounding the implant. PEG hydrogel implants in C57BL/6 mice have been shown to have similar foreign body response and cytokine activation in vivo as medical grade silicone (Lynn et al. 2010). When PEG hydrogels are formed into nanoparticles of differing elasticity, softer nanoparticles (10 kPa) had significantly reduced phagocytic uptake by murine macrophages (J774) than stiffer nanoparticles (3000 kPa), suggesting that regardless of size of PEG substrate, stiffer substrates induce greater inflammatory actions of macrophages compared to softer PEG substrates (Anselmo et al. 2015). However, this trend was both species and material specific. For instance, human macrophages (THP-1) showed enhanced TNF-α secretion on soft substrates (1.4 kPa) compared to stiffer ones (248 kPa) (Irwin et al. 2008). When compared side by side on polyacrylamide gels, RAW 264.7 murine macrophages and human U937 macrophages showed enhanced TNF-α secretion in response to softer gels in the presence of LPS (Patel et al. 2012). The variability in macrophage response to substrate stiffness may be due to the differences in other material properties and chemistries as well as the time at which response is analyzed (Brown et al. 2009; Brodbeck et al. 2002; Schutte et al. 2009).

Depending on where the biomaterial is being placed, macrophages surrounding or within the biomaterial may be exposed to a variety of mechanical factors that induce stretch. Stretch and shear stress elements are located in the promoter regions of pro-inflammatory genes, suggesting a role in modulating macrophage activation (Resnick and Gimbrone 1995; Shyy et al. 1995). For instance, primary peritoneal

Table 13.6 Mechanical factors influence macrophage activation

Mechanical factors	Material	Species	Model	In vitro/ in vivo	Macrophage response	Material type	References
Soft substrate	PEG	Mouse	C57BL/6	In vivo	M2	Hydrogel	Blakney et al. (2012)
	PEG	Mouse	C57BL/6	In vivo	M2	Hydrogel	Lynn et al. (2010)
	PEG	Mouse	J774	In vitro	M2	Nanoparticle	Anselmo et al. (2015)
	PEG	Human	THP-1	In vitro	M1	Interpenetrating polymer network	Irwin et al. (2008)
	Polyacrylamide	Mouse	RAW 264.7	In vitro	M1	Hydrogels	Patel et al. (2012)
	Polyacrylamide	Human	U937	In vitro	M1	Hydrogels	Patel et al. (2012)
Stiff substrate	PEG	Mouse	C57BL/6	In vivo	M1	Hydrogel	Blakney et al. (2012)
	PEG	Mouse	C57BL/6	In vivo	M1	Hydrogel	Lynn et al. (2010)
	PEG	Mouse	J774	In vitro	M1	Nanoparticle	Anselmo et al. (2015)
	PEG	Human	THP-1	In vitro	M2	Interpenetrating polymer network	Irwin et al. (2008)
	Polyacrylamide	Mouse	RAW 264.7	In vitro	M2	Hydrogels	Patel et al. (2012)
	Polyacrylamide	Human	U937	In vitro	M2	Hydrogels	Patel et al. (2012)
Static stretch	Collagen	Rat	Peritoneal macrophages	In vitro	M1	Coated wells	Wehner et al. (2010)
Cyclic stretch	Bioflex Silastic	Human	Alveolar derived	In vitro	M1	Coated wells	Pugin et al. (1998)
	Bioflex Silastic	Human	THP-1	In vitro	M1	Coated wells	Pugin et al. (1998)
	Poly-ε-caprolactone bisurea	Human	PBMC	In vitro	M1 to M2 with higher stretch	Strips	Ballotta et al. (2014)

macrophages from rats exposed to static stretch had elevated mRNA expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1β, and IL-6, suggesting that stretch induces pro-inflammatory or classical activation of the macrophage (Wehner et al. 2010). Unlike with substrate stiffness, the patterns with stretch are not material specific. For instance, human alveolar and THP-1 monocyte-derived macrophages exhibited high levels of IL-8 secretion in response to cyclic stretch compared to macrophages grown on materials under static conditions (Pugin et al. 1998). The stretch induced an even greater release of TNF-α and IL-6 when applied concurrently with LPS stimulation. The level of stretch within a material, however, is also important to macrophage polarization. For example, when compared to unstretched control, human PBMCs seeded onto poly-ε-caprolactone bisurea strips exposed to a 12% strain showed a higher shift of classically activated macrophages to the alternatively activated state over a 7-day period compared to those exposed to a 7% strain which showed higher alternative activation to classical activation polarization shift (Ballotta et al. 2014).

13.2.7 Protein Adsorption

Although many biomaterial properties influence macrophage response, the response to the property is generally best correlated with the proteins bound to a surface as well as the avidity with which the proteins bind to cells when on the surface. For this reason, protein adsorption onto the biomaterial is the most widely studied biomaterial parameter influencing macrophage activation (Table 13.7) (Anderson et al. 2008; Kastellorizios et al. 2015; Diekjiirgen et al. 2012; Walkey et al. 2012; Larson et al. 2012; Sanchez-Moreno et al. 2015; Yan et al. 2013; Jansch et al. 2013; Klopfleisch 2016). It is well understood that upon implantation, biomaterials quickly become coated with proteins from blood and surrounding fluid (Roach et al. 2005; Cross et al. 2016; Vogler 2012; Ihlenfeld and Cooper 1979). Adsorbed proteins differ based on biomaterial properties, but have the ability to modulate the cellular response to the material. The Vroman effect dictates what type of proteins become absorbed onto the surface and in what affinity they are adsorbed (Vroman and Lukosevicius 1964; Xu and Siedlecki 2007). Additionally, while on the surface, these proteins may undergo conformational changes, which may expose different bioactive sites of the protein for cells to interact with (Roach et al. 2005; Cross et al. 2016; Wilson et al. 2005; Gray 2004). While there remains a paucity of data on all protein interactions between biomaterial and macrophages, certain circulating proteins have made key impacts on modulating macrophage response, including fibronectin, von Willebrand factor, immunoglobulin G (IgG), albumin, vitronectin, and fibrinogen.

The blood-clotting protein, von Willebrand factor, for instance, inhibits the adhesion of monocytes and macrophages to biomaterials (Jenney and Anderson 2000b). With this inhibition, there is a decrease in macrophage binding to the biomaterial, and foreign body giant cell formation is inhibited (Anderson et al.

Table 13.7 Protein adsorption and macrophage activation

Protein	Material	Species	Model	In vitro/ in vivo	Macrophage response	Material type	References
von Willebrand factor	Glass	Human	PBMC	In vitro	M2	Plates	Jenney and Anderson (2000b)
Albumin	Polystyrene	Rat	Wistar	In vivo	M2	Nanospheres	Ogawara et al. (2004)
	Carbon	Mouse	RAW 264.7	In vitro	M2	Nanotubes	Dutta et al. (2007)
	Silicone	Mouse, Human	J774, THP-1	In vitro	M2	Nanoparticles	Parodi et al. (2013)
IgG	Plastek M	Human	PBMC	In vitro	M1	Plates	Jenney and Anderson (2000a)
	Polystyrene	Mouse	BMDM	In vitro	M1	Microbeads	Boyle et al. (2012)
	Polystyrene, Primaria	Human	PBMC	In vitro	M1	Plates	Shen et al. (2004)
Fibronectin	PEG	Mouse	C57BL/6 BMDM	In vitro	M1	Hydrogels	Lynn and Bryant (2011)
	mPEGmA	Rat	Sprague-Dawley	In vivo	M1	Implant	Kao and Lee (2001)
	Polystyrene	Mouse	C57BL/6	In vivo	M1	Microparticle	Zaveri et al. (2014)
Fibrinogen	PEG	Mouse	C57BL/6 BMDM	In vitro	M1	Hydrogels	Lynn and Bryant (2011)
	mPEGmA	Rat	Sprague-Dawley	In vivo	M1	Implant	Kao and Lee (2001)
	Polystyrene	Mouse	C57BL/6	In vivo	M1	Microparticle	Zaveri et al. (2014)
Vitronectin	PEG	Mouse	C57BL/6 BMDM	In vitro	M1	Hydrogels	Lynn and Bryant (2011)
	mPEGmA	Rat	Sprague-Dawley	In vivo	M1	Implant	Kao and Lee (2001)
	Polystyrene	Mouse	C57BL/6	In vivo	M1	Microparticle	Zaveri et al. (2014)

CD200	Polystyrene	Mouse	C57BL/6 BMDM	In vitro	M2	Microbeads	Kim et al. (2014)
IL-10	Nylon	Mouse	C57BL/6	In vivo	M2	Mesh	Higgins et al. (2009)
IL-4	PLGA	Rat	Lewis, BMDM	In vitro	M2	Microparticles	Minardi et al. (2016)
CD47	Polystyrene	Mouse	C57BL/6 BMDM	In vitro	None	Nanoparticle	Qie et al. (2016)

2008). Interestingly, macrophages have three von Willebrand factor receptors, which do not contribute to adhesion in the presence of the protein. This is in direct opposition to the adhesive properties of von Willebrand factor in other cells, such as endothelial cells (Carreno et al. 1993; Denis et al. 1993; Beacham et al. 1992).

Like von Willebrand factor, albumin has macrophage-repellant effects, which makes it useful in materials coatings for avoiding opsonization (Elzoghby et al. 2012; Orringer et al. 2009; Ogawara et al. 2004; Elsabahy and Wooley 2012). Synthetic nanoparticles coated with albumin have been shown to evade phagocytic activities of macrophages (Parodi et al. 2013). Additionally, albumin may actually inhibit inflammatory activities of macrophages when adsorbed onto the biomaterial. For instance, macrophages decreased COX-2 expression when exposed to single-walled carbon nanotubes with adsorbed albumin (Dutta et al. 2007).

On the other hand, surfaces coated with high levels of IgG tended to have enhanced adhesion of macrophages and high levels of pro-inflammatory cytokine release, including TNF-α (Jenney and Anderson 2000a; Boyle et al. 2012; Shen et al. 2004). IgG-coated surfaces also led to foreign body giant cell formation. This is thought to be due to complement receptors, which recognize IgG and may activate the opsonization process in macrophages (Shen et al. 2004; Brevig et al. 2005; Ademovic et al. 2006; Hu et al. 2001; Phillips and Kao 2005).

Other extracellular matrix proteins such as fibronectin, fibrinogen, and vitronectin facilitate macrophage adhesion and activation via integrin receptors on the macrophage. Materials coated with these proteins induce macrophage activation and foreign body giant cell formation. Using integrin-binding peptides, blocking integrin receptors on macrophages minimized inflammation and foreign body giant cell formation (Lynn and Bryant 2011; Kao and Lee 2001; Zaveri et al. 2014; Ratner and Bryant 2004).

Because of the importance of protein adsorption onto biomaterial and subsequent host response to the biomaterial modulated by this, some groups have taken advantage of this as a way to design implants and materials that evade the host immune response or promote positive outcomes for the implant. One example of this is macrophage activation inhibitory receptor, cluster of differentiation 200 (CD200), which has implications in modulating immune-mediated events including infection, allergy, autoimmunity, transplant response, and cancer progression (Nathan and Muller 2001; Liao et al. 2013). Coating materials with CD200 prior to subcutaneous implantation resulted in reduced macrophage activation and inflammatory response (Kim et al. 2014). Similarly, macrophage-deactivating cytokine (IL-4 or IL-10)-coated surfaces promoted tissue integrative and wound healing responses from macrophages in direct contact with the material (Higgins et al. 2009; Minardi et al. 2016). Coating with cluster of differentiation 47 (CD47), a member of the integrin-associated family, resulted in phagocytosis by macrophages due to integrin binding, but this did not influence macrophage polarization (Qie et al. 2016). The results of these studies and others illustrate that macrophages respond specifically to proteins coated on surfaces of biomaterials. It is potentially this interaction that is the final delineator of how a macrophage becomes activated in response to a biomaterial.

13.3 Conclusions

Recent advances in the literature have shown that macrophages have the ability to change polarization states and influence homeostatic and disease processes. Biomaterials differ in their innate properties that may induce a wide variety of responses in macrophages. Subsequently, these responses are key determinants of biocompatibility, controlling the foreign body reaction, and/or a tissue integrative and anti-inflammatory response. To elicit desired macrophage and immune responses to the biomaterial, several key components should be considered and tuned appropriately. Among these components are size, geometry, surface topography, architecture, hydrophobicity, surface chemistry, material mechanics, and protein adsorption. By manipulating biomaterial parameters, the material's innate properties may be used to control its overall destiny in the body. Ultimately, intelligent development and design of biomaterials will enhance desired biologic responses and activity.

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Chapter 14
Macrophage Differentiation in Normal and Accelerated Wound Healing

Girish J. Kotwal and Sufan Chien

Abstract Chronic wounds pose considerable public health challenges and burden. Wound healing is known to require the participation of macrophages, but mechanisms remain unclear. The M1 phenotype macrophages have a known scavenger function, but they also play multiple roles in tissue repair and regeneration when they transition to an M2 phenotype. Macrophage precursors (mononuclear cells/monocytes) follow the influx of PMN neutrophils into a wound during the natural wound-healing process, to become the major cells in the wound. Natural wound-healing process is a four-phase progression consisting of hemostasis, inflammation, proliferation, and remodeling. A lag phase of 3–6 days precedes the remodeling phase, which is characterized by fibroblast activation and finally collagen production. This normal wound-healing process can be accelerated by the intracellular delivery of ATP to wound tissue. This novel ATP-mediated acceleration arises due to an alternative activation of the M1 to M2 transition (macrophage polarization), a central and critical feature of the wound-healing process. This response is also characterized by an early increased release of pro-inflammatory cytokines (TNF, IL-1 beta, IL-6), a chemokine (MCP-1), an activation of purinergic receptors (a family of plasma membrane receptors found in almost all mammalian cells), and an increased production of platelets and platelet microparticles. These factors trigger a massive influx of macrophages, as well as in situ proliferation of the resident macrophages and increased synthesis of VEGFs. These responses are followed, in turn, by rapid neovascularization and collagen production by the macrophages, resulting in wound covering with granulation tissue within 24 h.

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14.1 Introduction

Wounds in aging or diabetic patients can be difficult to heal or can become chronic, resulting in considerable public health challenges. The development of a wound in response to an injury is followed by a healing process carried out predominantly by white blood cells (leukocytes) known as macrophages, which mediate phagocytosis to remove pathogens, as well as dead and dying cells, from the wound site. Besides this scavenger function, however, macrophages play multiple other roles in wound healing. Macrophages or their monocyte precursors infiltrate and reside in the wound site, where they are activated by pro-inflammatory cytokines, interferons, pathogen-associated modifying proteins (PAMPs), or damage-associated modifying proteins (DAMPs).

The two most predominant forms of macrophages are the M1 form, the classical activated form that acts in pathogen phagocytosis and destroys/removes damaged cells, including neutrophils, and the M2 form, which has repair and regeneration functions. The M1 to M2 polarization, or switch, reflects the macrophage differentiation process that shifts the cells from inflammation to proliferation functions. This polarization is a vital step in wound healing and is brought about by IL-4, IL-10, glucocorticoids, prostaglandins, and modulators of glucose and lipid metabolism (Landen et al. 2016). The M2 form resides in tissues and secretes anti-inflammatory mediators and growth factors, along with suppressors of cytokine-signaling (SOCS) proteins (Gordon et al. 2016), and it switches off the damage-causing pro-inflammatory activities of the M1 macrophages. The transition to the M2 form can be amplified by IL-4, and the increased numbers of M2 cells then result in elevated levels of IL-10, TGF-beta, and IL-12 (in minute quantities).

Both the M1 and M2 macrophages are critical for wound healing. During the natural wound-healing process (without intervention), blood-derived polymorphonuclear neutrophils enter the tissue, followed after 48 h by CXCR4-bearing mononuclear cells/monocyte precursors that transition to macrophages to become the major cells at the wound tissue site. The four-phase natural wound-healing process is characterized by a lag of approximately 3–5 days and requires an intermediate step of activation of resident fibroblasts and myofibroblasts during the remodeling phase for collagen production. Prolonged inflammation can result in fibrotic wound healing (Zhu et al. 2016).

More than 100 factors are known to lead to the formation of nonhealing wounds, such as diabetic foot ulcers (DFUs) (Valls et al. 2009; Davidson 1998; Ovington and Schultz 2004; Pugh and Ratcliffe 2003; Harding et al. 2002), but one known critical pathophysiology is the ischemia associated with a deficient blood supply (Silver 1980; Niinikoski et al. 1991; Ehrlich et al. 1972; Theoret 2005; Im and Hoopes 1970). Ischemia may not be the initiating factor for wounds like DFUs, because most DFUs start from a combination of neuropathy, pressure loading, infection, and/or trauma (Medina et al. 2005; Fang and Galiano 2008). However, tissue ischemia is the main hindrance to healing—wounds do not heal in tissue that

does not bleed, whereas they always heal in tissue that bleeds extensively (Gottrup 2002; Schaffer et al. 2002; Niinikoski et al. 1991).

The patterns of DFUs seen in Western countries have changed in the last two decades, with the neuroischemic ulcer now replacing the previously predominant neuropathic type as the most frequently seen DFU in many clinics (Boulton 2013). Strategies for increasing the wound oxygen supply with hyperbaric oxygen therapy have not shown consistent results (Berendt 2006; Cohen et al. 1999; Hunt and Pai 1972; Niinikoski 2003), because oxygen alone cannot adequately reverse ischemic damage. The most critical consequence of ischemia is a decrease in cellular energy supply (Im and Hoopes 1970; Smith et al. 1999), as energy is required for every aspect of the wound-healing process. For example, the linking of individual amino acids through peptide bonds to form a protein requires a significant input of chemical energy (Hunt and Pai 1972). Therefore, a protein such as collagen cannot be synthesized by fibroblasts (Fine and Mustoe 2001), nor can these cells proliferate, under ischemic or hypoxic conditions (Hunt and Pai 1972; Stadelmann et al. 1998). Other cellular processes, such as cell migration and proliferation, membrane transport, and growth factor production, also consume cellular energy and are also essential for wound healing (Wang et al. 1990). Thus, although small-scale clinical trials have shown the effectiveness of addition of growth factors such as bFGF to nonischemic wounds, these beneficial effects disappear in hypoxic dermal ulcer models (Harding et al. 2002; Lindblad 2007).

No one has yet studied the energy status of diabetic wounds. A Medline search retrieved only one article that reported ATP and PCr (phosphocreatine) contents in dorsal foot skin in 10 normal and 16 diabetic adults and noted a fourfold difference in the ATP/PCr ratio between diabetic and normal foot skin (Smith et al. 1999). We have measured the high-energy phosphate content in 34 diabetic and 21 postoperative wounds. Although both wounds had a similar appearance, we observed a two- to threefold decrease in the high-energy phosphate content of the diabetic wounds.

Ischemic wounds cannot be effectively treated by a direct infusion of free ATP because the bilayer cell membrane is impermeable to most water-soluble molecules (Cooper 1997). Specific transport proteins (carrier or channel proteins) permit the selective passage of small molecules across the membrane, but larger molecules, especially charged forms like ATP, cannot cross the cell membrane under normal conditions. An added complexity is that even when infused into the bloodstream, the half-life of ATP in the blood is less than 40 s, which poses technical difficulties for maintaining a stable ATP supply by intravenous administration (Puisieux et al. 1994).

These problems could be potentially overcome by encapsulating the ATP in specially formulated and highly fusogenic nanoscale unilamellar lipid vesicles (ATP vesicles or ATP nanoliposomes). If the chemical composition of the nanoliposome shell is similar to that of cell membranes, these vesicles will fuse with the cell membrane upon contact and deliver their ATP contents into the cytosol (Chien 2010). The current composition of the ATP nanoliposomes used in our research is 100 mg/ml L-α-phosphatidylcholine (Soy PC)/1, 2-Dioleoyl-3-trimethylammonium-propane (DOTAP) (50:1), Trehalose/Soy PC (2:1), 10 mM

KH2PO4, and 10 mM Mg-ATP. The diameters of the lipid vesicles range from 85 nm to 150 nm. The preparation can be freeze-dried and then reconstituted with deionized water and mixed with a neutral cream (Velvachol, Coria Laboratories, Fort Worth, TX) for use as a local wound dressing.

The use of nanoliposome-encapsulated Mg-ATP alters and accelerates the entire wound-healing process by triggering a novel pathway in which the activation and polarization of macrophages to the M2 form is absolutely critical and central (Howard et al. 2014; Kotwal et al. 2015). This novel alternative pathway is characterized by an increase in pro-inflammatory cytokines (TNF, IL-1 beta, IL-6), chemokine (MCP-1) release, activation of purinergic receptors (a family of plasma membrane receptors found in almost all mammalian cells), and increases in the numbers of platelets and platelet microparticles. This results in a massive influx of macrophages, as well as in situ proliferation of the incoming macrophages as early as 12 h after ATP-nanoliposome application, followed by increases in vascular endothelial growth factors that promote neovascularization and collagen production.

14.2 Infection Control by Macrophages in Natural and Assisted Wound-Healing Processes

Although the issue is still somewhat debated, macrophages are thought to play essential direct and indirect roles in the complicated wound-healing process (Martin and Leibovich 2005). This process requires that any microbial contamination of the wound site be eliminated by antimicrobial action and by phagocytosis and opsonization by macrophages. Phagocytosis takes place when macrophage receptors recognize PAMPs on the surfaces of bacteria or fungi, prompting the macrophages to attach to and engulf the invading microbes. A phagolysosome is then formed inside the macrophage by the fusion of the engulfed bacterium with the lysosome. This is followed by a proteolytic breakdown of the bacterium and the release of the breakdown material by exocytosis. The process of opsonization, by contrast, is triggered by an activation of the alternate complement pathway, due to recognition of the lectins and sugar chains on bacterial surfaces. This then results in deposition of the breakdown products of the third complement component onto the microbe surfaces. Macrophages have receptors for these breakdown products (C3b and iC3b), so they attach specifically to bacteria bearing the complement C3b and iC3b. This initiates opsonization, which is phagocytosis due to the specific targeting of macrophages to bacteria. Besides infection control, the role of macrophage phagocytosis is to clear cell components that are formed after cell death; these components have their own molecular patterns, called DAMPs.

The classical wound-healing process, which includes hemostasis, inflammation, proliferation, and remodeling, is characterized by a 3–5 day lag (Delavary et al. 2011). During the inflammatory phase, pro-inflammatory cytokines (IL-1beta, IL-6,

and TNF alpha) are secreted to recruit peripheral circulating white blood cells and monocytes, which differentiate into macrophages. Stimulation with interferon gamma and lipopolysaccharide causes classically activated macrophages to polarize into cytotoxic phenotypes. During the reepithelialization stage, while phagocytosing surrounding dead cells and debris, macrophages secrete vascular endothelial growth factors and promote the proliferation of endothelial cells, skeletal myoblasts, and fibroblasts, while also secreting IL-10 to suppress further influxes of macrophages. This phase is followed by angiogenesis, myotube formation, and collagen production. The final remodeling phase involves collagen remodeling (Novak and Koh 2013).

14.3 The Role of Alternately Activated Macrophages in Accelerated Wound Healing

Extensive studies on mice now indicate a temporal variability during the wound-healing process, which suggests that selected phenotypic traits are promoted during the classically activated early phase of wound healing, followed by later alternatively activated phases. This aspect of wound healing has been reviewed by Brancato and Albina (2011), who suggest that the lack of a pure macrophage form in vivo indicates that activated macrophages could appropriately be described as a continuous spectrum of distinct and continuous phenotypic features. The wound macrophages express the alternatively activated M2 phenotype, so they have been proposed to represent *wound-healing macrophages*.

14.4 Macrophage Phenotype Switching

The macrophage phenotype changes as the wound heals, progressing from the M1 (pro-inflammatory) to the M2 (anti-inflammatory) forms, indicating that the wound-healing phenotype is influenced by the local microenvironment (Ferrante and Leibovich 2012). The M2 phenotype is characterized by distinct surface receptors or cell differentiation (CD) markers (CD68, CD163, CD206) and shows several subphenotypes (M2a, M2b, M2c, and M2d). The switch from the M1 to the M2 phenotype occurs in stages in response to downregulation of IL-10 and upregulation of IL-4 and IL-13 and in response to specific mediators. The M1 to M2a transition is induced by activation by IL-4R-alpha.

Macrophage polarization, which is the process that leads to the acquisition of distinct phenotypes, must occur in an orderly manner for effective wound healing. Macrophage polarization is regulated by a complex process in which adenosine-mediated "switching" regulates angiogenesis during the final stages of the repair process that results in wound healing (Ferrante and Leibovich 2012). Another layer of complexity is added by the presence of specific macrophage phenotypes associated with each tissue type (Davies et al. 2013a, b).

14.5 Accelerated Wound Healing Following Intracellular ATP Delivery

In the past half century, numerous interventions have been proposed for either facilitating or accelerating the wound-healing process (Garash et al. 2016; Ogle et al. 2016). In sharp contrast to the classical wound-healing process that occurs with all presently used interventions, the fast-track process occurs in response to the direct introduction of nanoliposome-encapsulated Mg-ATP to the wound. This intracellular ATP delivery via fusogenic unilamellar nanoliposomes induces a significant wound-healing enhancement in the skin of rodent models (Chiang et al. 2007). This enhanced healing was reaffirmed in a rabbit model, where full-thickness skin wounds treated with ATP nanoliposomes showed accelerated wound sealing as well as extremely rapid tissue regeneration. The traditional 3–6 day lag was eliminated, and granulation tissue started to appear within 24 h, a phenomenon never observed previously or reported with any other therapeutic treatment modality.

The early growth is composed mainly of macrophages, suggesting active proliferation. Collagen is produced, neovascularization is enhanced at the wound site, and re-epithelializing tissue tunnels through the granulation tissue (Wang et al. 2009). The top of the granulation tissue ultimately peels off, revealing a healed wound. A similar growth pattern is not observed following treatment with either unencapsulated Mg-ATP, empty nanoliposomes, or with the only FDA-approved prescription growth factor for wound dressing—Regranex. Most notably, the growth has a self-limiting characteristic, so that it leaves no hypertrophic scars or any other unusual growth, even 2 years after the treatment (Howard et al. 2014).

This type of healing contrasts sharply with the conventional process typically observed by those engaged in wound care, where fibrin, platelets, and erythrocytes are the major components of the provisional matrix seen early after injury. This matrix is then gradually replaced by granulation tissue during a proliferation phase that begins after a 3–5-day lag (Levenson and Demetriou 1992; Schaffer et al. 2002).

The individual steps that lead to this novel wound-healing process have been assessed by testing several individual activities triggered by the delivery of ATP (Howard et al. 2014). In vitro studies showed an increase in the chemokine MCP-1, accompanied by a reduction in the anti-inflammatory cytokine IL-10 levels, in human macrophage cells treated with ATP nanoliposomes (Wang et al. 2009). These chemokine and cytokine changes could explain the large influx of macrophages observed following ATP-nanoliposome administration (Kieran et al. 2013). The predominant macrophage phenotype was M2, which was consistent with the observed increase in IL-13R and the detection of the CD163 cell differentiation surface marker (Howard et al. 2014). The level of vascular-specific growth factors also increased, consistent with the observed neovascularization (Chiang et al. 2007). Early increases were also observed in platelet numbers at the wound site (Wang et al. 2010). The inference made at the time was that the influx,

accumulation, and phenotypic differentiation of the incoming stem cells and macrophages were a result exclusively of the increased ATP energy supply (Chien 2006, 2010).

Recent studies have shed more light on the mechanism underlying this rapid wound healing in response to ATP. The rapid granulation and the accompanying tissue generation and macrophage proliferation (as determined by increased PCNA synthesis and BrdU staining) appear to drive collagen synthesis without an intermediate fibroblast generation at the wound site (Howard et al. 2014). These multiple activities seen in response to treatment with nanoliposome-encapsulated ATP are summarized below. Previous studies by Wang et al. (2009) indicated that the phenomenon of macrophage accumulation in response to the ATP-nanoliposome treatment could be attributed to the ATP-driven rearrangement of the chromatin structure within the 12-component cell proliferation SWI/SNF complex, which includes the BRG1 or Brm ATPase subunits. They also suggested that the increased levels of BRG1 and BRM in the wounds treated with ATP nanoliposomes could be associated with ATPase activity that contributed to the in situ proliferation of the macrophages, thereby suggesting a possible mechanism for the fast-track wound-healing pathway (Yang et al. 2015). The current understanding of the mechanism of wound healing following intracellular ATP delivery illustrated in Fig. 14.1 can be summarized as follows:

1. Stem/progenitor cell recruitment, in response mostly to purinergic receptor activation.
2. Leukocyte chemotaxis toward the wound.
3. Enhanced platelet accumulation at the wound site.
4. Monocyte accumulation and activation from processes 1 to 3; the platelet-derived growth factors further enhance monocyte activation.
5. Monocyte transformation to macrophages via platelets and platelet microparticles and the MCP-1 pathways.
6. Massive cell accumulation caused by in situ macrophage proliferation.
7. Changes in RNA expression patterns to generate building blocks to support proliferation.
8. Activated macrophages perform their phagocytic functions, but also secrete MCP-1, resulting in further cell accumulation.
9. Macrophages activated by the alternate pathway also produce collagen directly in response to nanoliposome-encapsulated ATP. This process results in much faster collagen production than normally occurs indirectly by fibroblasts.
10. Upregulated apoptosis keeps the growth in check and maintains the balance between proliferation and regression.

The elucidation of the steps involved in the proliferation phase that follows the inflammation phase after an injury will provide a better understanding of the rapid wound-healing mechanism associated delivery of nanoliposome-encapsulated ATP. Other possibilities could include the increased expression of small RNAs (Kotwal and Chien, unpublished) and other building blocks that would facilitate M2 macrophage proliferation.

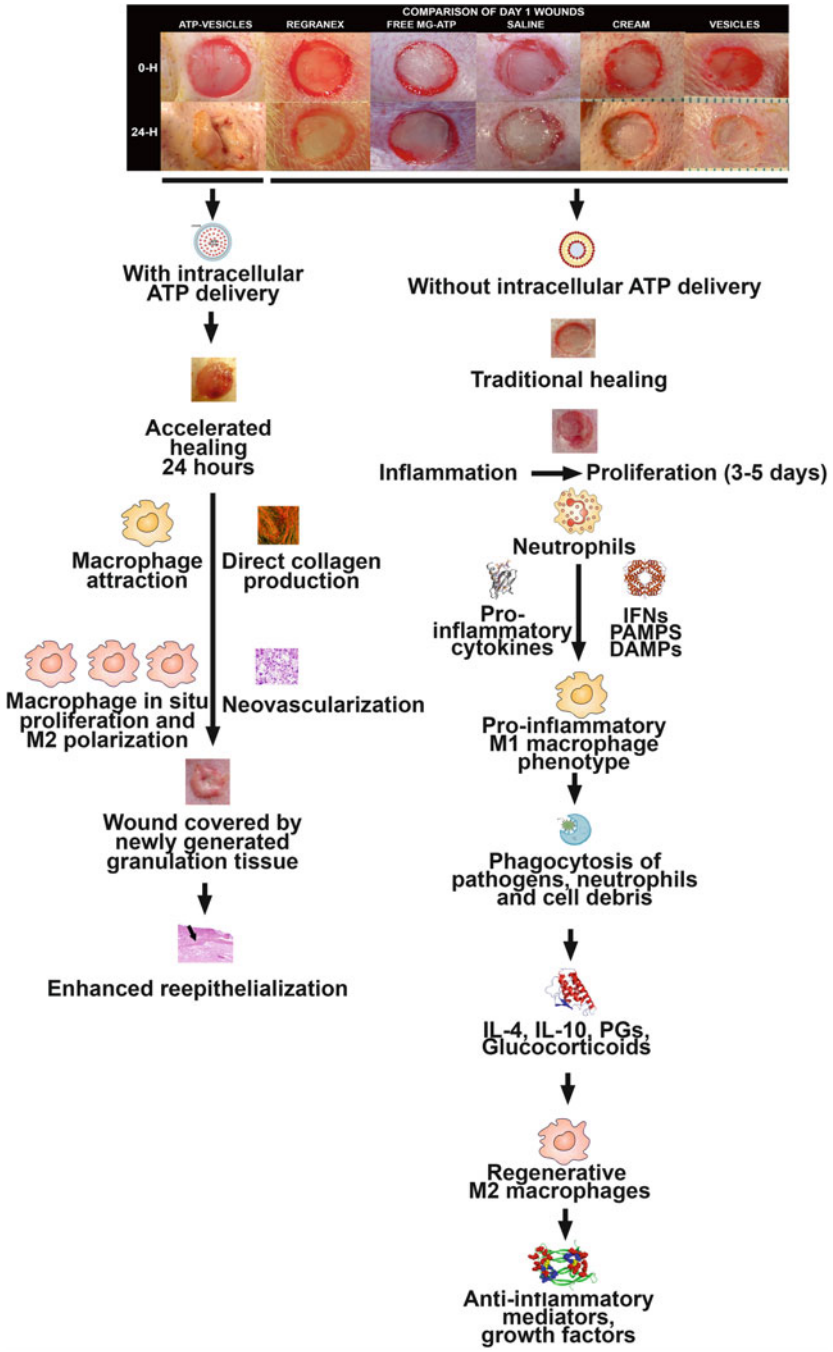

Fig. 14.1 Flow diagram of Steps following wound treatment with or without Intracellular ATP delivery

14.6 Regulation of Macrophage Polarization

Autoimmune damage can theoretically occur from the immune system itself due to unregulated complement activation or phagocytic killer cell activation, such as is induced by M1 macrophages. Therefore, timely regulation of these activations is critical. The complement system has multiple regulatory proteins that can bind key complement proteins and limit the release of chemotactic factors that normally draw an influx of macrophages and pro-inflammatory cells. The transition from inflammation to proliferation also requires some form of control or regulatory system that regulates macrophage activation.

The macrophage polarization and soluble mediator gene expression occurring during inflammation are under epigenetic control (Kapellos and Iqbal 2016). Specifically, M1 or M2 activation occurs by the posttranslational modifications of DNA-binding histones found adjacent to genes encoding proteins that function as inflammatory response mediators. The posttranslational modifications include methylations and acetylations catalyzed by methyltransferases, dimethyltransferases, acetyltransferases, and deacetyltransferases. These posttranslational modifications contribute to the epigenetic control of macrophage polarization to either the M1 or M2 phenotypes, which, in turn, modulates the type of cytokines and chemokines that are generated and the types of immune cells that are present at the wound site.

14.7 Conclusion

Wound healing is a complex process driven by the actions of macrophages (Snyder et al. 2016). The inflammatory phase occurs following the influx of neutrophils, and then monocytes, and their activation to macrophages of the inflammation, or M1, phenotype. These M1 macrophages then transition to the M2, or the proliferative phenotype, and the M2 macrophages then drive the natural wound-healing process. Extensive studies in a rabbit wound model developed by the Chien group have indicated that the pathway of wound healing that occurs in response to treatment with nanoliposome-encapsulated ATP is unique, as gross healing commences within 24 h via macrophages that proliferate and then transform directly into the extracellular matrix to rapidly fill in the wound cavity. This contrasts strongly with the natural form of wound healing, where gross healing starts only after about a 3–6-day lag, and the wound space is filled with a provisional matrix consisting of red cells trapped in a fibrin mesh. The elimination of the lag time and the very early ability to support cell survival and proliferation in a wound cavity without any blood supply are two features never achieved with any other wound treatment.

The rapid tissue generation following intracellular ATP delivery is a major clinical advance that awaits full elucidation of its detailed mechanism. At present, the possible explanations for the hastened wound healing include the provision of

energy to the wound microenvironment, the overexpression of noncoding RNAs such as 5S rRNA and U4 splicesome RNA (Kotwal and Chien, unpublished) that contribute to a more efficient buildup of the biochemical building blocks needed for proliferation, the adenosine-mediated macrophage phenotype switching, or all of the above.

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Chapter 15
Macrophages and RhoA Pathway in Transplanted Organs

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Abstract RhoA is a small GTPase that, via its downstream effectors, regulates a variety of cell functions such as cytokinesis, cell migration, vesicular trafficking, and phagocytosis. As such the RhoA pathway is also pivotal for proper functioning of immune cells including macrophages. By controlling actin cytoskeleton organization, RhoA pathway modulates macrophage's polarity and basic functions: phagocytosis, migration, and extracellular matrix degradation. Numerous studies indicate that macrophages are very important effectors contributing to acute and chronic rejection of transplanted organs. In this review we discuss the role of RhoA pathway in governance of macrophage's functions in terms of transplanted organs.

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15.1 Introduction

For thousands of patients with terminal organ failure, the only cure is organ transplantation. After transplantation every transplant has some degree of ischemia reperfusion injury caused by blood returning to the graft after a period of lack of oxygen (ischemia) (Kloc et al. 2014; Mannon 2012; Salehi and Reed 2015). The acute rejection of transplanted organs, which occurs within days to months after transplantation and is mainly T cell dependent, is manageable through genetic matching and immunosuppression. However, there is no cure for the long term, occurring within months to years posttransplantation, chronic rejection that is mainly macrophage dependent (Julius et al. 2000; Lamb et al. 2011; Nasr et al. 2016; Sa et al. 2016). Around 70% of transplanted organs are lost within 10 years posttransplantation due to chronic rejection. Thus, finding the cure for chronic rejection is one of the most important challenges in organ transplantation. The most striking symptoms of chronic rejection are the occlusion (formation of neointima) of graft arteries (graft vascular disease, GVD) and tissue fibrosis, both of which are dependent on the activity of macrophages recruited to the graft (Kloc and Ghobrial 2014; Liu et al. 2016c; Skaro et al. 2002; Schnoor et al. 2008). GVD and destruction of graft tissue integrity lead to the ischemia and failure of the graft (Kloc and Ghobrial 2014). Because the recruitment of macrophages and their functions depend on the RhoA pathway, the macrophages and the RhoA pathway have recently emerged as possible targets for therapeutic intervention in both chronic rejection and ischemia reperfusion injury (Kloc et al. 2014; Mannon 2012; Salehi and Reed 2015).

RhoA (Ras homolog gene family, member A) belongs to the Rho GTPase family of hydrolases that bind and hydrolyze guanosine triphosphate (GTP). This family of proteins contains three subfamilies: Rho, Rac (Ras-related C3 botulinum toxin substrate), and Cdc42 (cell division control protein 42 homolog). These small proteins switch their activation state by binding to either GTP (the active form) or GDP (the inactive form). In the GTP-bound state, these proteins interact with the downstream effectors to elicit a variety of intracellular responses (Maekawa et al. 1999). Studies of various cell types have indicated that Rho regulates the assembly of actin and actomyosin filaments, while Rac and Cdc42 modulate the polymerization of actin to form peripheral lamellipodial and fillopodial protrusions. RhoA is a multifunctional protein that through its downstream partners regulates transcription, protein secretion and vesicle trafficking, and a plethora of actin-dependent processes relevant to cell migration, including cell-substrate adhesion and cell-cell adhesion (Riento and Ridley 2003). Among RhoA downstream effectors, ROCKs (ROCK1 and ROCK2, Rho-associated, coiled-coil-containing protein kinases 1 and 2) are implicated in cell adhesion and smooth muscle contraction. As serine-threonine protein kinases, ROCKs phosphorylate myosin light chain (MLC) phosphatase and inhibit its activity, which in turn leads to an increase in MLC phosphorylation and consequently increases actin-based contractility (Uehata et al. 1997). In addition, ROCK, through activating LIM-kinase (LIMK), phosphorylates cofilin, which has

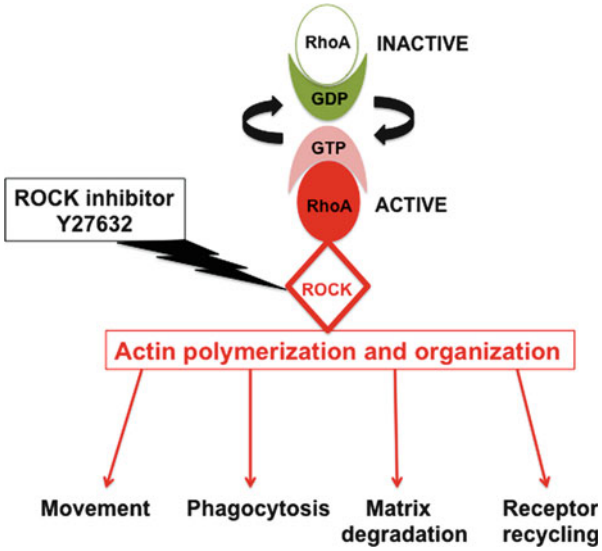

Fig. 15.1 Macrophage functions regulated by RhoA pathway. RhoA is a small GTPase and its downstream effector is kinase ROCK (two isoforms Rock1 and ROCK 2). RhoA/Rock are master regulators of actin polymerization and organization. Through actin RhoA/ROCK regulate major actin-dependent macrophage functions such as movement, phagocytosis, matrix degradation, and receptor recycling. One of the RhoA pathway inhibitor used in rodent transplantation models is Y27632 that strongly inhibits ROCK1 and weakly inhibits ROCK2

actin-depolymerizing activity (Dawe et al. 2003; Worthylake et al. 2001; Worthylake and Burridge 2003). These functions are sensitive to Y-276232, a specific inhibitor of ROCK. In vitro studies using HeLa cells have shown that overexpression of LIM-kinase induced the formation of actin stress fibers in Y-27632-sensitive manner (Maekawa et al. 1999). In chronic rejection models, several laboratories showed that administration of Y-27632 to the graft recipient inhibits chronic rejection in rodent cardiac allografts (Kloc et al. 2014; Ohki et al. 2001; Tharaux et al. 2003; Zhang et al. 2013). Our group showed that Y-27632 affects mouse macrophage phenotype/polarity, phagocytosis, migration, and matrix degradation (Figs. 15.1 and 15.2; Liu et al. 2016d).

15.2 RhoA Regulation of Cell Movement and Matrix Degradation

In non-muscle cells, RhoA/ROCK pathway is involved in several physiological processes including migration (Figs. 15.1 and 15.2; Riento and Ridley 2003). It has been shown that in monocytes and prostate cancer cells, ROCKs regulate cell

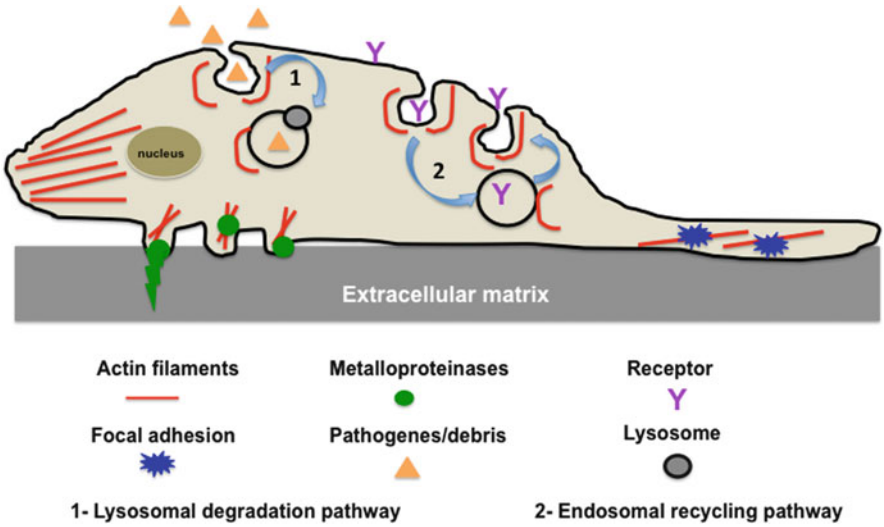

Fig. 15.2 Macrophage functions dependent on RhoA and actin cytoskeleton. Migrating macrophage becomes polarized into front and tail region. The movement forward is dependent on actin filaments arrangement and cyclic assembly/disassembly of vinculin-rich focal adhesions in the tail. During movement within the tissues, macrophage uses podosomes to degrade the extracellular matrix. Podosomes are actin-rich extension of ventral (*bottom*) macrophage membrane. Podosomes contain metaloproteinases that are delivered to the podosomes on microtubules and digest extracellular matrix. Macrophage phagocytosis is used for degradation of pathogens and cell debris and for recycling of various membrane-bound surface molecules such as cell surface receptors. The formation of phagocytic cup and subsequent formation of phagosome are actin dependent. The formation of phagolysosome and vesicles in lysosomal degradation pathway (1) or endosomal recycling pathway (2) is also actin dependent. Internalized pathogens and cell debris, which are destined for destruction, enter lysosomal degradation pathway. Internalized molecules such as receptors, which are destined for recycling and coming back to the membrane, enter endosomal pathway

migration by enhancing actomyosin contractility (Somlyo et al. 2000; Worthylake et al. 2001). The movement of macrophages forward (the amoeboid type of movement) requires sequential attachment and detachment/retraction of cell tail from the surface. The attachment/detachment process is regulated by ROCK and relies on sequential assembly and disassembly of actin and vinculin-rich focal adhesions (Fig. 15.2; Parsons et al. 2010). Studies from our laboratory showed that mouse macrophages treated with ROCK inhibitor Y27632 or macrophages with deleted RhoA gene are unable to disassemble focal adhesions in the tail. This inability to retract the tail causes macrophages to elongate (they acquire so-called hummingbird phenotype) and extend "indefinitely" until the breaking point (Liu et al. 2016a, b, d) and inhibits their migration into the graft (Liu et al. 2016b).

The migration of macrophages into the graft depends not only on the amoeboid movement but also on the ability to penetrate tissues, which, in turn, requires degradation of extracellular matrix. Macrophages contain podosomes, a specialized

matrix-degrading organelle. The podosomes are membrane protrusions located on the ventral (bottom) surface of the macrophage and are required for the multidirectional migration through cell barriers and the interstitium. They contain bundles of actin filaments and metaloproteinases-rich vesicles, which facilitate proteolysis of extracellular matrix (Fig. 15.2; El Azzouzi et al. 2016; Meddens et al. 2016). It has been shown that podosomes contain the active form of RhoA (Varon et al. 2006) and that inactivation of RhoA by dominant negative RhoA inhibitor C3 exoenzyme disrupts the podosome structure (Berdeaux et al. 2004).

15.3 RhoA Regulation of Phagocytosis

A crucial of macrophages is phagocytosis that capacitates their role of defenders against infectious pathogens and cleaners of cell and tissue debris. Phagocytosis occurs primarily through two main processes: the Fcγ receptor (which binds the IgG antibodies) and the integrin complement receptor 3 (CR3)-mediated actin-dependent processes (Fig. 15.2; Aderem and Underhill 1999; Fuentes et al. 2014; Gray and Botelho 2017; Naj and Linder 2016; Stow and Condon 2016). The CR3 complex (complement receptor 3 (CR3) (CD11b/CD18) is a pattern recognition receptor that binds a variety of bacterial molecules. RhoA pathway is known to be involved in the phagocytosis mediated by CR3 processes (Kim et al. 2012). An inhibitor of the Rho GTPases, C3 toxin fused with Tat peptide (Tat-C3 toxin), was shown to reduce filamentous actin and inhibit the phagocytosis of serum-opsonized zymosans (Kim et al. 2012). In addition, Olazabal's group found that inhibition of the RhoA/ROCK pathway caused a decreased accumulation of Arp2/3 complex (the actin-related proteins ARP2 and ARP3 serve as nucleation sites for new actin filaments) and F-actin around opsonized phagocytized particles, possibly leading to the reduction in CR-mediated phagocytic engulfment (Olazabal et al. 2002). Therefore, inhibiting RhoA pathway is believed to decrease the phagocytosis ability of macrophages. From the transplantation perspective, the efficient phagocytosis, by eliminating inflammation-inducing cell debris, is beneficial for transplant well-being and survival. It has been shown that inhibition of phagocytosis at the time of transplantation accelerates rejection of cardiac allografts (Snawder et al. 2013). This indicates that inhibition of RhoA pathway has to be carefully balanced because it can be both beneficial (inhibition of macrophage migration) and harmful (inhibition of phagocytosis) for transplant survival.

15.4 RhoA Pathway in Allograft Rejection

Macrophage accumulation has long been recognized as a feature of allograft rejection. As an important component of innate immunity, macrophages attract more and more attention in acute and chronic rejection field of research.

Immediately after transplantation, the activated tissue-resident macrophages and blood-borne macrophages (which enter during ischemia reperfusion) mount defensive responses such as phagocytosis of necrotic debris, secretion of pro-inflammatory cytokines, and production of reactive nitrogen and oxygen (Liu et al. 2016c). Though their exact role in allograft rejection remains obscure, any disturbance of macrophage function may alter the outcome of transplantation.

It has been shown in acute kidney rejection model that the percentage of recipient-derived macrophages is dramatically elevated, indicating that the migrating recipient monocytes are a crucial source of macrophages that infiltrate the graft (Rugtveit et al. 1996). Once monocytes/macrophages infiltrated the allograft during ischemic reperfusion, they display a pro-inflammatory phenotype by secreting inflammatory cytokines, interleukins (IL-1β, IL-12, IL-18), tumor necrosis factor alfa (TNF-α), and interferon gamma (IFN-γ), and directly damage graft tissue (Liu et al. 2016c). The posttransplantation treatment with carrageenan (seaweed-derived sulfated polysaccharide) depletes 30–80% of macrophages reducing graft GVD. This indicates that macrophages play a major role in the development of GVD (Kitchens et al. 2007). In addition, macrophages are able to produce collagen and, thus, directly contribute to tissue fibrosis (Schnoor et al. 2008). Recently, Syrjälä and colleagues showed that the treatment of donor heart with (COMP)-Ang1, a chimeric form of vascular growth factor angiopoietin1 (Ang1), and cartilage oligomeric matrix protein (COMP), reduces expression of RhoA and limits ischemia reperfusion injury, reduces influx of macrophages and neutrophils, and abrogates rejection of cardiac allografts (Syrjala et al. 2015).

15.5 RhoA-Dependent Recruitment of Macrophages into the Transplant

Donor macrophages recruited to the transplanted organ originate from the circulating monocytes (Epelman et al. 2014). The monocytes in the bloodstream are recruited to the transplanted organs through binding of their appropriate receptors to the chemoattractants/chemokines secreted by graft tissue and its vascular endothelium (Fig. 15.3). There are three main recruitment pathways: (1) the chemokine (C-C motif) ligand 3 (CCL3)/macrophage inflammatory protein-1α (MIP-1α) that binds to the CCR1, CCR4, and CCR5 receptors (Grau et al. 2000), (2) the monocyte chemoattractant protein-1 (MCP-1/CCL2) that binds to CCR2 and CCR4 receptors (Grandaliano et al. 1997), and (3) the chemokine (C-X3-C motif) ligand 1, CX3CL1/fractalkine that binds to CXCR3 receptor (Fig. 15.3; Li et al. 2008). It has been shown that in atherosclerotic plaque development and mouse cardiac transplant settings, the preferable pathway used by monocytes/macrophages is the CX3CL1/CX3CR1 fractalkine pathway (Liu et al. 2016b; Tacke et al. 2007). Barlic

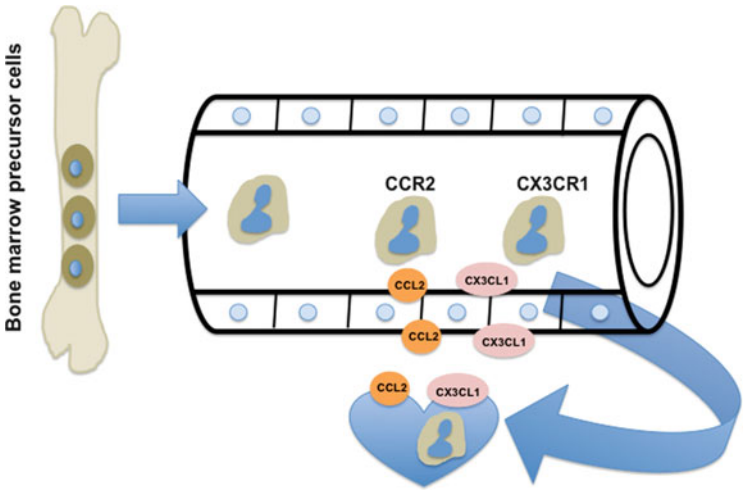

Fig. 15.3 Monocyte/macrophage recruitment to the transplant. The monocytes produced from bone marrow precursors and entering circulation express receptors such as CCR2 and fractalkine CX3CR1 for various chemoattractants/chemokines such as CCL2 and CX3CL1 that are produced by inflamed tissues or endothelium of transplant's vessels. The binding of cytokines to the appropriate receptors on the surface of monocytes recruits them to the target tissue/organ, where they differentiate into tissue macrophages

et al. (2006) described the phenomenon of CCR2 to CX3CR1 receptor switching in human coronary artery. This explains how by inhibiting CCR2-dependent recruitment in favor of CX3CR1-dependent pathway macrophages and monocytes are able to select between recruitment pathways to accumulate in the vessel wall. Recently, we showed that macrophage-specific deletion of RhoA inhibited monocyte/macrophage infiltration, neointimal hyperplasia of vasculature, and abrogated chronic rejection of the mouse cardiac allografts (Liu et al. 2016b). We also showed that RhoA deletion caused downregulation of fractalkine receptor CX3CR1 by disruption of its recycling, which relies on actin-dependent endosomal pathway (Figs. 15.2 and 15.3; Liu et al. 2016b). We proposed the following scenario of how a RhoA deletion and downregulation of CX3CR1 receptor lead to the inhibition of chronic rejection of cardiac allograft in mouse model. It is known that the CX3CR1 receptor belongs to the family of G-coupled proteins that have the ability to change into the guanine nucleotide exchange factors (GEFs). The GEFS are proteins that activate GTPases (including RhoA GTPase) by stimulating the exchange between guanosine diphosphate (GDP) and guanosine triphosphate (GTP). Upon binding to CX3CL1 (which is produced by arterial wall endothelium), the CX3CR1 changes into GEF that activates RhoA (Fig. 15.4). This, in turn, influences actin polymerization in monocytes/macrophages and promotes their migration to artery walls and

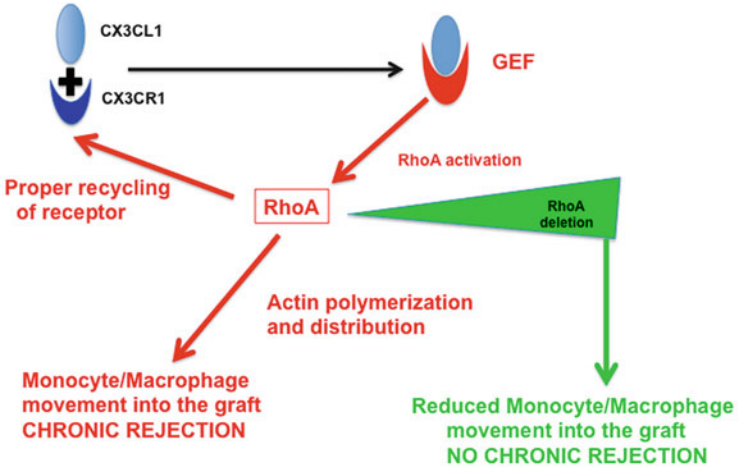

Fig. 15.4 RhoA/actin/CX3CR1 signaling interactions. The fractalkine CX3CR1 receptor expressed on the surface of monocytes and macrophages binds its ligand CX3CL1 cytokine produced by endothelium of transplant's vessels. Upon ligand binding the CX3CR1 that is a G protein-coupled receptor changes confirmation and becomes the guanine nucleotide exchange factor (GEF) that is able to activate RhoA. Activated GTP-bound RhoA induces changes in actin polymerization and distribution status, which in turn lead to monocyte/macrophage migration and recruitment into the transplanted organ, while in the transplant, the macrophages elicit inflammatory response, vessel occlusion, and transplant rejection. Active RhoA also allows for actin-dependent proper recycling of CX3CR1 receptors. When RhoA is deleted from monocytes/macrophages, the receptor recycling is inhibited; this leads to decreased expression of CX3CR1 receptors, decreased RhoA activation, and decreased recruitment of monocytes/macrophages to the graft and leads to inhibition of chronic rejection. Parts of the figure were modified from Liu et al. (2016b)

culminates in development of neointima and vessel blockade and leads to chronic rejection (Liu et al. 2016b). The deletion of RhoA in monocytes/macrophages affects actin-dependent endosomal recycling of CX3CR1 receptors, which inhibits expression of CX3CR1 and inhibits their recruitment to the transplanted heart (Figs. 15.4 and 15.5; Liu et al. 2016b). Decreased abundance of macrophages in the transplant reduces neointimal hyperplasia of blood vessels and results in inhibition of chronic rejection (Fig. 15.5; Liu et al. 2016b). Based on this finding, we postulate that targeting the RhoA and the macrophage recruitment CX3CR1 (fractalkine) pathway can be potentially used as anti-chronic rejection intervention in clinical trials.

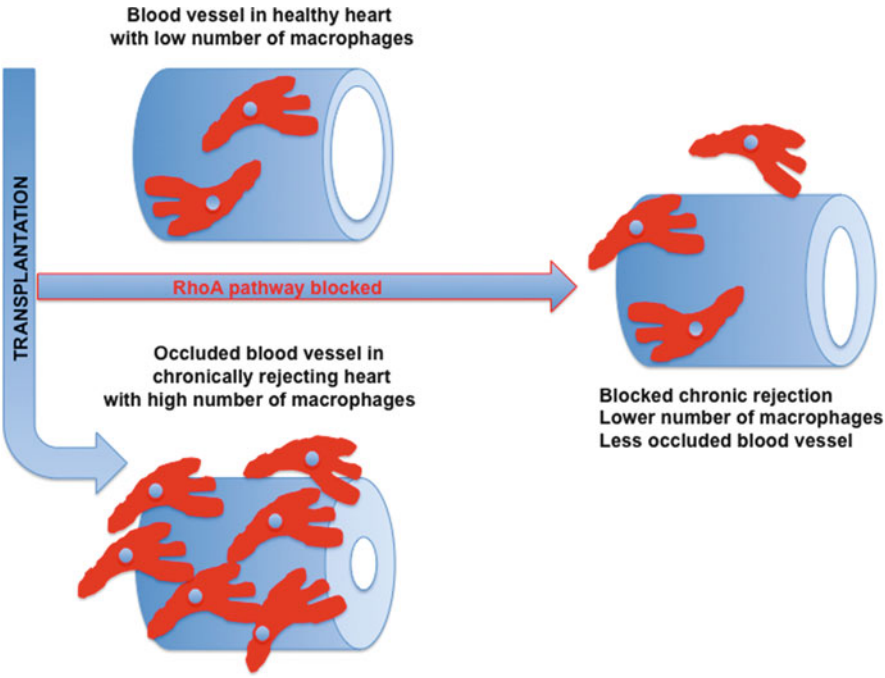

Fig. 15.5 RhoA pathway inhibition abrogates chronic rejection of transplant. Healthy heart has low level of macrophages and normal non-occluded blood vessels. Upon transplantation macrophages accumulate around the blood vessels and induce neointima formation, vessel blockade, and fibrosis, which eventually lead to chronic rejection of the graft. Disruption of RhoA pathway (either by Y27632 inhibition of ROCK or RhoA-deletion) reduces number of monocytes/ macrophages, which are able to enter the graft resulting in the inhibition of chronic rejection

15.6 Conclusions

A growing number of studies indicate that RhoA pathway is pivotal for proper functioning of immune response. By controlling actin cytoskeleton organization, RhoA/Rock modulates macrophage's polarity and basic functions: phagocytosis, migration and extracellular matrix degradation. Although much more comprehensive studies are necessary to answer how RhoA pathway modulates macrophages' behavior, current data indicate that inhibition of RhoA pathway may be a potential therapeutic method for transplant rejection.

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