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Origins and Hallmarks of Macrophages: Development, Homeostasis, and Disease

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Preface

Macrophages the most plastic cells of the hematopoietic system are found in all tissues and exhibit great functional diversity. They have roles in development, homeostasis, tissue repair, and immunity. While anatomically distinct, resident tissue macrophages exhibit different transcriptional profiles, and functional capabilities, they are all required for the maintenance of homeostasis. However, these reparative and homeostatic functions can be subverted by chronic insults, resulting in a causal association of macrophages with disease states. In this review, we discuss how macrophages regulate normal physiology and development and provide several examples of their pathophysiologic roles in disease. We define the “hallmarks” of macrophages performing particular functions, taking into account novel insights into the diversity of their lineages, identity, and regulation. This diversity is essential to understand because macrophages have emerged as important therapeutic targets in many important human diseases.

Introduction and Perspectives

Macrophages, originally identified by Metchnikoff by their phagocytic nature, are ancient cells in Metazoan phylogeny. In adult mammals, they are found in all tissues where they display great anatomical and functional diversity. In tissues, they are organized in defined patterns with each cell occupying its own territory, a type of tissue within a tissue. Although several attempts have been made to classify macrophages, the most successful definition is the mononuclear phagocytic system (MPS), which encompasses these highly phagocytic cells and their bone marrow (BM) progenitors. In the MPS schema, adult macrophages are defined as end cells of the mononuclear phagocytic lineage with tissue macrophages deriving from circulating monocytes that originate in the BM. However, this definition is inadequate as macrophages have several origins during ontogeny and each of these different lineages persist into adulthood where they display great diversity [1]. Other classifications have included binary classifications that refer to inflammatory states. These include the activated (AM) and alternatively activated (AAM) or M1 or M2 [2,3] defined by responses to

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the cytokines IFNγ and activation of TLRs and IL4/IL13 respectively. While this is a useful heuristic that may reflect extreme states such as in AMs in THI mediated immune responses or AAM in parasitic infections [2] it cannot represent the complex in vivo milieu for most macrophage types where numerous cytokines and growth factors interact to define the final differentiated state of macrophages. Indeed transcriptional profiling of resident macrophages by the "Immunological Genome Project" find that these populations show great transcriptional diversity with minimal overlap suggesting many unique classes[1].

Macrophages have roles in almost every aspect of an organism's biology ranging from development, homeostasis, to repair through to immune responses to pathogens. Resident macrophages regulate tissue homeostasis by acting as sentinels and responding to changes in physiology as well as challenges from outside. During these homeostatic adaptations, macrophages of different phenotypes can also be recruited from the monocyte reservoirs of blood, spleen and bone marrow [4], and perhaps, from resident tissue progenitors or through local proliferation [5,6]. Unfortunately, in many cases, these homeostatic and reparative functions can be subverted by continuous insult, resulting in causal association of macrophages with disease states, such as fibrosis, obesity and cancer (Figure 1). Macrophages, therefore, are an incredibly diverse set of cells constantly shifting their functional state to new set points in response to changes in tissue physiology or environmental challenges. They should not even be considered as one cell type but be subdivided into different functional subsets with acknowledgement to their different origins.

This review will not dwell on macrophage responses to pathogens that have been discussed extensively by others[2,7,8], but will emphasize the homeostatic mechanisms by which macrophages contribute to physiological and pathophysiologic adaptations in mammals. It will define the hallmarks of "macrophage" performing particular functions, taking into account new insights into the diversity of their lineages, identity and regulation. This phenotypic diversity is essential to understand because macrophages are central to many disease states, and have emerged as important therapeutic targets in many diseases.

Macrophage Origins Rewritten

Ontologically, the MPS has been proposed to arise from a rigid temporal succession of macrophage progenitors [9]. In mice, these first develop at embryonic (e) day 8 from the primitive ectoderm of the yolk sac and give rise to macrophages that do not have a monocytic progenitor. This primitive system is followed by definitive hematopoiesis in the fetal liver, which is initially seeded by hematopoietic progenitors from the yolk sac and subsequently from the hematogenic endothelium of the aorto-gonadal-mesonephros region of the embryo. Thereafter, during embryogenesis the fetal liver is the source of definitive hematopoiesis generating circulating monocytes. Coincident with the post-natal formation of bone, fetal liver hematopoiesis declines and is replaced by BM hematopoiesis. This definitive hematopoiesis is the source of circulating monocytes (resident Ly6c- and inflammatory Ly6c+ in mice) and from which it has been considered that all resident macrophages in tissues are derived [4]. However, this model for the formation of the MPS has been challenged (Figure 2). First, lineage-tracing experiments have shown that Langerhans cells (LC) and microglia are primarily derived from the yolk sac progenitors while LCs have a mixed origin from YS and fetal liver [10,11]. Second, even in the absence of hematopoietic stem cells, yolk sac progenitors were capable of giving rise to the major tissue resident population of macrophages (defined as F4/80 bright) in skin, spleen, pancreas, liver, brain and lung. In a few tissues, such as kidney and lung, macrophages were shown to have a chimeric origin (HSC- and yolk sac-derived), whereas other cells, such as classical dendritic cells (DC) and lesser populations of F4/80^{low} cells, were continuously replaced by BM-derived progenitors [6]. These data indicate that there are at least three lineages of

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macrophages in the mouse arising at different stages of development and persisting in the adult. It also calls into question the function of circulating monocytes because, at least in mice, these cells do not seed the majority of the adult tissues with macrophages. In fact, complete loss of $CD16^+$ monocytes in humans appears to be of little consequence [12]. Thus the function of monocytes needs to be defined with the possibility that patrolling monocytes (Ly6c-) act to maintain vessel integrity and to detect pathogens while inflammatory monocytes (Ly6c+) are recruited only to sites of infection or injury or to tissues that have continuous cyclical recruitment of macrophages such as the uterus.

Regardless of their origin, genetic and cell culture studies indicate the major lineage regulator of almost all macrophages is the colony stimulating factor 1 receptor, CSF1R. This class III transmembrane tyrosine kinase receptor is expressed on most, if not all, mononuclear phagocytic cells, and a reporter mouse expressing GFP from the *Csf1r* locus illustrates their relative abundance (5–20% of cells) and tissue distribution [13]. *Csf1r* expression and its requirement for differentiation distinguish macrophages from many but not all DCs sub-types [14]. Targeted ablation of the *Csf1r* causes severe depletion of macrophages in many tissues, such as brain, skin, bone, testis, ovary etc. Moreover, an initial comparison of the *Csf1r* null mice with a spontaneous mutant that lacks its cognate ligand CSF1 (*Csf1*$^{op/op}$ mice) demonstrated that all phenotypes in the receptor null were also found in the ligand null, indicating that CSF1 only has a single receptor [15]. However, the phenotype of the *Csf1r* null mice is more severe than that of the *Csf1* null mice, including the complete loss of microglia and LCs [10,16] in the *Csf1r* null mice, which suggested the presence of another ligand. Indeed, IL34, with a distinct but overlapping pattern of expression with *Csf1*, was recently identified as an additional ligand for the CSF1R [17]. Targeted ablation of *Il34* resulted in loss of microglia and LCs, but had little impact on BM, liver, or splenic macrophages [18].

Despite the importance of the CSF1R in macrophage specification, *Csf1r* null mutant mice still have some tissue macrophages, such as in the spleen, indicating the existence of other macrophage growth factors. Potential candidates include GM-CSF and IL3 that act as macrophage growth factors in tissue culture. However, mice lacking GM-CSF or IL3 do not exhibit significant defects in their tissue macrophages except alveolar ones that are regulated by GM-CSF [19]. VEGF is another candidate regulator of macrophages because it can compensate for the loss of *Csf1* in osteoclast development in vivo [20]. In contrast to CSF-1 that is found in all tissues and serum and is a basal regulator of macrophage number through a negative feedback loop [15], GM-CSF is not a steady-state ligand and appears to be synthesized in response to challenge [21]. GM-CSF and FLT3L regulate the maturation of DC populations with the notable exception of LCs, whose development is dependent on *Csf1r* [22]. Recent genomic profiling of LCs place them closer to macrophages than DCs and this data together with their lineage dependence on *Csf1r*, may require changes in classification [14]. DCs will not be discussed further in this review but their biology and lineages have been extensively covered recently [14].

In their basal state, resident tissue macrophages show great diversity in their morphologies, transcriptional profiles, anatomical locations, and functional capabilities [23]. This functional heterogeneity likely results from the dynamic crosstalk between resident tissue macrophages and the client cells that they support. To understand this macrophage diversity there must be an understanding of transcriptional regulation. The most important of these transcription factors is PU1, *Spl1*, a member of the ETS family whose loss following targeted mutation results in complete depletion of Cd11b+ F4/80+ macrophages including those derived from the YS [6]. However, *Pu.1* action is not limited to macrophages as B cells are also severely depleted in these null mutant mice. Similarly other members of the ETS family are also involved in macrophage differentiation including *Ets2* that positively regulates the *Csf1r*

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promoter. In adults, *Mafb* (*c-Maf*) is required for the local proliferation that maintains resident macrophages [4]. In the differentiation of osteoclasts, *Fos* and *Mitf* are required [24], whereas *Gata-2* is also required for monocyte development but not resident macrophage populations [25]. However, little is known about the transcriptional control of the differentiation of the diverse tissue macrophages, such as those in the liver and brain [13]. Rather most research has focused on their functional activation in response to environmental challenges [23], topics that will be discussed in following sections. Nevertheless, the recent transcriptional profiling of resident macrophages has identified many candidate transcription factors including those that may regulate core macrophage-associated genes such as *MiT* (Micropthalmia) family members, *Tcef3*, *C/EBPa*, *Bach1*, *Creg-1* and those unique to sub-populations including *Gata6* and *Spi-c* whose targeted gene ablation will undoubtedly define sub-sets of macrophages and their unique activities [1].

Macrophages in Development

Metchnikoff proposed that macrophages participate in the maintenance of tissue integrity and homoeostasis. This necessitated that macrophages be able to discriminate self from non-self, sense tissue damage, and recognize invading pathogens, an insight that led to the concept of innate immunity for which he was awarded the Nobel Prize. The properties that included sensing inside from out together with motility throughout the organism, phagocytosis and degradation were later sequestered to instruct the acquired immune system as it evolved to more efficiently deal with ever changing pathogenic challenges. This enhanced sophistication of the immune system probably resulted in the evolution of DCs as specialized mononuclear phagocytes to interface with the acquired immune system. Indeed, in mammals, DCs appear to be focused upon initiating tissue immune responses, while tissue macrophages on homeostasis and tissue integrity [9].

Emphasis on the immunological and repair aspects of macrophage function has rather overshadowed their importance in the development of many tissues. For instance, studies of *Csf1* null mutant mice, which lack many macrophage populations, have revealed a cluster of developmental abnormalities [19]. Most notable amongst them is the development of osteopetrosis, which is caused by the loss of bone reabsorbing macrophages known as osteoclasts. This phenotype, which is also observed in *Spi1* null mice, is axiomatic for the roles of macrophages in development, in that cell fate decisions are unchanged but the tissue remodeling and expression of growth factors is lost. Specifically, while bone formation is intact in *Csf1* or *Spi1* null mice, the bones are not sculpted to form the cavities where hematopoiesis can ensue [19]. Consequently, the functional integrity of the bones, in terms of load bearing and hematopoiesis, is compromised. *Csf1* null mice survive to adulthood because of extra-medullary hematopoiesis in the spleen and liver [19], and as mice age, osteoclastogenesis is rescued by compensatory expression of VEGF [20].

Remodeling deficiencies in the absence of macrophages have also been noted in several other tissues, including the mammary gland, kidney and pancreas suggesting a general requirement for macrophages in tissue patterning and branching morphogenesis [19,26]. In the mammary gland, the best studied of these tissues, macrophages are recruited to the growing ductal structure and their loss results in a slower rate of outgrowth and limited branching, phenotypes that are re-iterated during the mammary growth caused by pregnancy [19]. This stems in part from the failure to remodel the extracellular matrix during the outgrowth of the ductal structures. However, recent studies have also implicated macrophages in maintaining the viability and function of mammary stem cells, which reside at the tip of the duct known as the terminal end bud and are responsible for the outgrowth of this structure [27]. Similar roles for macrophages in stem cell biology has been suggested in the maintenance of intestinal integrity and its regeneration after damage [28], whereas a sub-population of

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macrophages in the hematopoietic niche regulates the dynamics of hematopoietic stem cell release and differentiation [29]. Furthermore in regenerating livers, macrophages specify hepatic progenitor fate through the expression of WNT ligands and antagonism of Notch signaling [30]. Macrophage control of stem cell function is clearly an emerging and important research area.

As professional phagocytes, macrophages perform critical functions in the remodeling of tissues, both during development and in the adult animal. For instance, during erythropoiesis, maturing erythroblasts are surrounded by macrophages, which ingest the extruded erythrocyte nuclei. Remarkably, this function of macrophages is critically important because in its absence, erythropoiesis is blocked and lethality ensues [31]. Macrophages also make decisions about hematopoietic egress from the bone marrow through engulfing cells that do not express the CD47 ligand [32]. They also maintain hematopoietic steady state via engulfment of neutrophils and erythrocytes in the spleen and liver and the failure of this activity results in neutropenia, splenomegaly and reduced body weight [33]. Phagocytosis particularly of apoptotic cells is obviously central to macrophage function and this is emphasized by the build up in macrophage-depleted mice of such cells during development for example during the resolution of the inter-digit areas during limb formation [34]. However, there is no apparent consequence to this phenomenon as non-professional phagocytes clear excess apoptotic cells. Despite this, macrophages have evolved to “eat” cells, and to suppress inflammation and auto-immunity in response to self-antigens that might arise during homeostasis [35].

Macrophages also regulate angiogenesis through a variety of mechanisms. This has been most extensively studied in the eye during its development. Early in the postnatal period during regression of the hyaloid vasculature, macrophages identify and instruct vascular endothelial cells (VEC) to undergo apoptosis if these cells do not receive a counter-balancing signal from pericytes to survive. Wnt7b synthesized by macrophages delivers this cell death signal to the VECs and either its absence or the absence of macrophages, there is vascular over-growth [36]. WNT secretion is also required later in retinal vasculature development but in this case macrophage synthesized Wnt5a, a non-canonical WNT, induces expression of soluble VEGFR1 through an autocrine mechanism that titrates VEGF and thereby reduces vascular complexity so that the vascular system is appropriately patterned [37]. Furthermore, at other times of ocular development macrophages regulate vascular complexity. In this circumstance macrophage synthesized VEGFC reinforces Notch signaling [38]. In addition, during angiogenesis in the hindbrain macrophages enhance the anastomosis of tip and stalk cells to give functional vessels [39]. These macrophage functions are not restricted to the vascular arm of the circulatory system since they also play roles in lymphangiogenesis during development [40] and in adults they play a significant role in maintaining fluid balance through their synthesis of VEGFC [41].

Brain development is also influenced by macrophages. These macrophages called microglia depend on CSF1R signaling for their presence [10,16]. In the absence of this signaling there are no microglia and the brains of these mice have significant structural defects as they mature [16]. Both CSF1 and IL34 are expressed by neurons in a mutually restricted pattern of expression with IL34 being the major factor for microglial differentiation and viability [10,42]. The disruption of architecture in the *Csf1r* null mouse brain together with well-documented deficiencies in neuronal processing regulating olfaction and the reproductive axis in the hypothalamus in *Csf1* null mice strongly suggest that microglia are involved in the development of neuronal circuitry and maintenance of brain structure [16,19]. Indeed microglia have been shown to promote neuron viability [19], modulate neuronal activity [43], to prune synapse during development [44] as well as express a range of neuronal growth and survival factors including NGF [19]. This conjecture is reinforced because a hypomorphic mutation in

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the *CSF1R* in humans is responsible for hereditary diffuse leukoencephalopathy with spheroids that results from loss of myelin sheaves and axonal destruction [45]. These trophic activities of microglia are also consistent with macrophage roles in neuroprotection following injury in a variety of contexts. These effects include the promotion of survival and proliferation of retinal progenitor cells and the regeneration of adult sensory neurons [46–48]. However, caution needs to be exercised in attributing all the phenotypes observed in the brains of *Csf1r* mutant mice or humans to the loss of microglia as *Csf1r* expression has been reported on neuronal stem cells and their development in vivo is regulated by CSF1R [42]. Nevertheless, it seems likely that microglia play important roles in the development of neuronal circuitry though their effects on the proliferation, survival and connectivity of neurons [43] or through effects on myelination or by modulating angiogenesis and fluid balance in the brain [16].

The examples cited above indicate a few of the roles for macrophages in normal development and these are likely to expand with further study. Phenotypically in mice, macrophages are all Cd11b+, CD68+ CSF1R+ F4/80+ and phagocytic and their activities are through the temporal and spatial delivery of developmentally important molecules such VEGFs and WNTs as well as proteases. These developmental roles of macrophages are re-capitulated in repair as described below but are also intimately involved in chronic conditions that lead to pathologies as well as the development and progression of malignancies.

Macrophages in Metabolic Homeostasis

Mammalian metabolic organs like liver, pancreas, and adipose tissue are composed of parenchymal and stromal cells, including macrophages, which function together to maintain metabolic homeostasis [49]. By regulating this interaction, mammals are able make dramatic adaptations to changes in their environment and nutrient intake. For instance, during bacterial infection, innate activation of macrophages results in secretion of pro-inflammatory cytokines, such as TNFα, IL6 and IL1β, which collectively promote peripheral insulin resistance to decrease nutrient storage[50,51]. This metabolic adaptation is absolutely necessary for mounting an effective defense against bacterial and viral pathogens because nearly all activated immune cells preferentially utilize glycolysis to fuel their functions in host defense. However, this adaptive strategy of nutrient reallocation becomes maladaptive in the setting of diet-induced obesity, a state characterized by chronic low-grade macrophage-mediated inflammation[51,52]. In the sections below, we provide a general framework for understanding the pleiotropic functions performed by macrophages to maintain metabolic homeostasis (Figure 3). Although our current knowledge in this area is primarily derived from studies in obese insulin resistant mice, it is likely that tissue resident macrophages also participate in facilitating metabolic adaptations in healthy animals.

White adipose tissue

White adipose tissue (WAT) is not only the principal site for long-term storage of nutrients but also regulates systemic metabolism via release of hormones termed “adipokines”[53]. These metabolic functions of WAT are primarily performed by adipocytes with trophic support provided by stromal cells, including macrophages. In this scenario, macrophage representation in WAT, both in terms of numbers and their activation state, reflects the metabolic health of adipocytes[51]. For instance, in lean healthy animals, adipose tissue macrophages comprise 10–15% of stromal cells and express the canonical markers ($Arg1^+$, $CD206^+$, $CD301^+$) of AAMs [54]. In contrast, macrophage content increases to 45–60% during obesity [55,56], resulting from increased recruitment of $Ly6C^{hi}$ monocytes that differentiate into inflammatory macrophages, as judged by their expression of *Nos2*, *Tnfa*, and *Itgax* [54,55]. Although these macrophages contribute to development of insulin resistance

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in adipocytes, recent studies suggest that these cells also participate in remodeling of the enlarging WAT, functions that facilitate the storage of excess nutrients in adipocytes [57]. This suggests that two macrophage subsets coordinate homeostatic adaptations in adipocytes of lean and obese animals.

In healthy animals, AAMs are critical for maintaining insulin sensitivity in adipocytes[51]. This trophic effect of AAMs is, in part, mediated by secretion of IL-10, which potentiates insulin action in adipocytes [54]. These observations led various groups to focus on cell-intrinsic and extrinsic mechanisms that control alternative activation of adipose tissue macrophages. For cell-intrinsic factors, transcription factors downstream of IL-4/IL-13 signaling, such as PPARγ, PPARδ, and KLF4, were found to be required for the maintenance of AAMs in WAT and metabolic homeostasis [58–61]. The dominant cell extrinsic factors regulating maturation of AAMs in lean WAT are the type 2 cytokines, IL-4 and IL-13 [60]. Absence of eosinophils, which constitute the major cell type capable of IL-4 secretion in WAT [62], impairs alternative activation of adipose tissue macrophages and makes mice susceptible to obesity-induced insulin resistance. Together, these reports have established that homeostatic functions performed by AAMs in WAT are required for metabolic adaptations to excessive nutrient intake.

While adipocytes in lean animals can easily accommodate acute changes in energy intake, chronic increase in energy intake places adipocytes under considerable metabolic stress. Consequently, the enlarging WAT releases chemokines, such as CCL2, CCL5, and CCL8, to recruit $Ly6C^{hi}$ inflammatory monocytes into the WAT [63], where these cells differentiate into $CD11c^{+}$ macrophages and form “crown-like structures” around dead adipocytes [54,64]. As these $CD11c^{+}$ macrophages phagocytize dead adipocytes and become lipid engorged, they initiate expression of inflammatory cytokines, such as TNF-α and IL-6, which promote insulin resistance in the surrounding adipocytes [54]. Presumably, this initial decrease in adipocyte insulin sensitivity is an adaptation to limit nutrient storage. However, in the setting of unabated increase in caloric intake, this adaptive response becomes maladaptive, contributing to pathogenesis of obesity-induced systemic insulin resistance.

Brown Adipose Tissue

In mammals, brown adipose tissue (BAT) is the primary thermogenic organ that is activated by exposure to environmental cold [65]. For decades, it had been thought that hypothalamic sensing of cold triggers an increase in sympathetic nerve activity to stimulate the BAT program of adaptive thermogenesis [65]. However, recent work has demonstrated a requirement for resident macrophages in facilitating the metabolic adaptations of brown and white adipose tissues to cold. Specifically, exposure to cold temperatures results in alternative activation of BAT and WAT macrophages, which are required for induction of thermogenic genes in BAT and lipolysis of stored triglycerides in WAT [66]. Accordingly, mice lacking AAMs are unable to mobilize fatty acids from WAT to maximally support BAT thermogenesis, which is necessary for the maintenance of core body temperature in cold environments. These supportive functions of macrophages are mediated by their secretion of norepinephrine, which surprisingly accounts for ~50% of the catecholamine content of BAT and WAT in the cold. Thus, cold-induced alternative activation of BAT and WAT macrophages provides an example of how resident macrophages provide trophic support to facilitate the function of tissue parenchymal cells, in this case the white and brown adipocytes.

Liver and Pancreas

Liver integrates nutrient, hormonal, and environmental signals to maintain glucose and lipid homeostasis in mammals. Over the last few years, evidence has emerged that Kupffer cells,

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the resident macrophages of liver, facilitate the metabolic adaptations of hepatocytes during increased caloric intake. During obesity, an imbalance between the uptake, synthesis, and oxidation of fatty acids results in increased lipid storage in hepatocytes, a key factor in the development of hepatic insulin resistance [67]. Interestingly, Kupffer cells directly participate in this process by regulating the oxidation of fatty acids in hepatocytes. An early insight into this process came from studies that identified PPARδ as an important regulator of IL-4 and IL-13 driven program of alternative macrophage activation [58,61]. These studies revealed that loss of PPARδ in myeloid cells specifically impaired alternative activation of Kupffer cells, resulting in hepatic steatosis and insulin resistance. A similar phenotype was observed when Kupffer cells were depleted in rodents using gadolinium chloride or clodronate-containing liposomes [68] Although the precise factors elaborated by Kupffer cells are still not known, co-culture studies suggest that Kupffer cell-derived factors work in *trans* to maintain hepatic lipid homeostasis [58,61].

Pancreas functions as an endocrine and exocrine gland in mammals. Recent findings suggest that, analogous to obesity-induced WAT inflammation, high fat feeding induces the infiltration of macrophages into the insulin-producing islets. In this case, the increased intake of dietary lipids results in β-cell dysfunction, which induces the expression of chemokines, such as CCL2 and CCCL1, to recruit inflammatory monocytes/macrophages into the islets [69,70]. Consequently, the secretion of IL1β and TNFα by the infiltrating macrophages augments β-cell dysfunction, resulting in impaired insulin secretion and hyperglycemia in obese mice. Although these reports have elucidated the pathogenic role of macrophages in β-cell dysfunction, in the future it will be important to determine whether macrophages also participate in the physiologic regulation of β-cell biology as they do during development and pregnancy [19].

Macrophages in disease

When tissues are damaged following infection or injury, inflammatory monocytes (Ly6c+ in mice) are recruited from the circulation and differentiate into macrophages as they migrate into the affected tissues [4]. These recruited macrophages often exhibit a pro-inflammatory phenotype in the early stages of a wound healing response. They secrete a variety of inflammatory mediators including, TNFα, IL1, and nitric oxide (NO), which activate anti-microbial defense mechanisms, including oxidative processes that contribute to the killing of invading organisms [7]. They also produce IL12 and IL23, which direct the differentiation and expansion of anti-microbial T_H1 and T_H17 cells that help drive inflammatory responses forward [3]. Although these inflammatory macrophages are initially beneficial because they facilitate the clearance of invading organisms, they also trigger substantial collateral tissue damage because of the toxic activity of reactive oxygen and nitrogen species and of T_H1 and T_H17 cells [71]. Indeed, if the inflammatory macrophage response is not quickly controlled, it can become pathogenic and contribute to disease progression, as is seen in many chronic inflammatory and autoimmune diseases [72,73]. To counteract the tissue damaging potential of the inflammatory macrophage response, macrophages undergo apoptosis or switch into an anti-inflammatory or suppressive phenotype that dampens the pro-inflammatory response, while facilitating wound healing [7]. These regulatory macrophages often produce ligands associated with development such as WNT ligands, that are essential for tissue repair [74]. It is becoming increasingly clear that the mechanisms that regulate the transformation of inflammatory macrophages into an anti-inflammatory cell or suppressive macrophages back into a proinflammatory phenotype has a major impact on the progression and resolution of many chronic diseases, as discussed in the following sections (Figure 4).

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Macrophages in Cancer

Tumors are abundantly populated by macrophages [3]. Originally thought to be part of an anti-tumor response, clinical and experimental data indicate that in the large majority of cases macrophages promote tumor initiation, progression and metastasis [75]. Macrophages in response to persistent infections or chronic irritation, synthesize inflammatory cytokines, IFNγ, TNFα, IL6, that engage other immune cells to sustain the chronic inflammation that appears to be causal in tumor initiation and promotion [76]. The tumor-inducing activities are multi-factorial for example, through the production of inflammatory cytokines such as IFNγ in UV-induced skin cancer [77], TNFα in carcinogen induced cancer, as well as by generating a mutagenic environment [76,78] or through alterations of the microbiome [79]. However, once tumors become established they educate the tumor associated macrophages (TAMs) away from an immunologically active state to adopt a trophic immunosuppressive phenotype that promotes tumor progression to malignancy [75].

In established tumors TAMs stimulate tumor cell migration, invasion, intravasation as well as the angiogenic response required for tumor growth [75,80,81]. These events are required for tumor cells to become metastatic by facilitating their escape into the circulatory or lymphatic system. Evidence from autochthonous models of breast cancer suggests that the macrophages take on these activities in response to CSF1 and IL4/IL13 encountered in the tumor microenvironment. For example, IL4 mediated differentiation [80] results in a reciprocal paracrine dialog between CSF-1 synthesized by tumor cells and EGF by TAMs that promotes tumor cell invasion and intravasation in mammary cancer [82]. In mammary cancers this loop is initiated by CXCL12 in the Polyoma Middle T-induced (PyMT) model or Heregulin in the Her2/Neu model. In human xenograft models, CCL18 is also required for tumor cell invasion and metastasis through triggering integrin clustering [83]. TAMs also remodel the tumor microenvironment through the expression of proteases such as matrix metalloproteinases (MMPs), cathepsins and uPA and matrix remodeling enzymes including lysyl oxidase and SPARC [81,84]. The proteases, such as Cathepsin B, MMP2, 7 and 9 cleave ECM providing conduits for the tumor cells as well as release of growth factors such as HB-EGF and EGF mimics that foster tumor cell invasion and metastasis [84,85].

Macrophages play an important role in tumor angiogenesis regulating the dramatic increase in vascular density known as the angiogenic switch required for the transition to the malignant state [86]. These angiogenic TAMs are characterized by the expression of the angiopoietin receptor, TIE2, similar to that found in macrophages during development [87,88]. Ablation of this specific population inhibits tumor angiogenesis and thus tumor growth and metastasis in a variety of models [87,88]. TAMs secrete many angiogenic molecules including VEGF family members, TNFα, IL1β, IL8, PDGF and FGF [75,88,89]. Of these myeloid derived VEGF is required for the angiogenic switch [89] but other aspects of angiogenesis can be independent of VEGF and involve the secreted protein Bv8 [90]. Angiogenic macrophages can be recruited to the tumors by hypoxia [88,91] but also by growth factors such as CSF1 and VEGF [92].

Tumors have a proclivity to metastasize to particular sites and this phenotype is partially defined by macrophages. Data suggest that the tumor produced fragments of ECM molecules or exosomes prepare these sites, known as pre-metastatic niches, to be receptive to the circulating tumor cells through recruitment of myeloid cells characterized by CD11b and VEGFR1 positivity [93,94]. These niches are tumor type dependent and the fate of the tumor cells can be reprogrammed to a different tissue by transfer of tumor-conditioned serum to a naïve mouse strain [93]. These niches are also dependent on coagulation as this is necessary for recruitment of the myeloid cells that have recently been more precisely defined as F4/80+ macrophages [95]. At lung metastatic sites mini-clots form that allow the arrest of tumor cells [95] that then produce CCL2 to recruit CCR2+ Ly6c+ inflammatory

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monocytes that rapidly develop into Ly6c- metastasis-associated macrophages (MAMs) [96]. These monocytes and MAMs promote tumor cell extravasation in part through their expression of VEGF that induces local vascular permeability. MAMs that are in intimate association with the tumor cells, also promote their viability via clustering of tumor cell expressed VECAM1 that interlocks with the MAM expressed counter receptor integrin a4 [83]. MAMs also promote subsequent growth of the metastatic cells and importantly ablation of these cells after the metastases are established inhibits metastatic growth [75].

In mice these individual pro-tumoral functions are performed by different sub-populations albeit expressing canonical markers such as CD11b, F4/80 and CSF1R [75]. This view is consistent with recent profiling of immune cells both in various tumor types in mice and human that indicates both differences in the extent of macrophage infiltrate as well as in phenotype [97]. For example detailed profiling in human hepatocellular carcinoma shows various macrophage sub-types defined by specific location that have both pro- and anti-tumoral properties through their engagement of the acquired immune system although overall the balance is tilted towards pro-tumoral functions [98]. Transcriptional profiling of TAM sub-populations in mice suggest they more closely resemble embryonic macrophages than inflammatory ones, being enriched in expression of developmentally relevant molecules such as those of the WNT pathway [75]. This strongly suggests that the trophic roles of macrophages found during development, in metabolism and in the maintenance of homeostasis are subverted by tumors to enhance their growth, invasion and complexity. However, transcriptional control of these different phenotypes is only just being revealed especially in vivo [3]. Many studies have analyzed macrophage response to LPS signaling via NFkB but this results in “activated” macrophages that largely are involved in anti-bacterial responses and likely to be anti-tumoral [23]. In contrast, TAMs in their trophic and immunosuppressive functions are shaped by IL-10 and IL4/13 that signal to STAT3 and STAT6 respectively [3,99]. PARPs and KLF4 also cooperate to induce a range of gene expression associated with their phenotype [3]. CSF1R in macrophages also signals to a wide range of transcriptional factors including classic ones such and MYC and FOS [15]. MYC signaling has been shown to be important for pro-tumoral phenotypes [100]. In turn, CSF1R expression is regulated by ETS2 transcription factors and genetic ablation of this factor in macrophages in PyMT tumors recapitulates the loss of CSF1 in tumors by inhibiting angiogenesis and decreased tumor growth [101]. The interaction of these factors together with other regulatory molecules such as miRNAs and epigenetic controls [3] will require sophisticated genomic analysis that will help differentiate the regulation of the multiple sub-sets [23]. These functions and other regulatory systems have been thoroughly reviewed recently [3].

Macrophages in inflammatory disease

Macrophages play major roles in many chronic diseases including atherosclerosis, asthma, inflammatory bowel disease, rheumatoid arthritis, and fibrosis[7,102–104]. In these conditions their contribution varies greatly in different situations and is controlled by many factors. For example, allergic asthma is a complex chronic inflammatory disease of the lung defined by airway inflammation, airway obstruction, airway hyper-responsiveness and pathological lung remodeling. The inflammatory response is characterized by the recruitment of T_H2 lymphocytes, mast cells, eosinophils, and macrophages to the lung and by elevated expression of allergen-specific IgE in the serum. It has been suggested that the chronicity of type-2 cytokine-mediated airway inflammation that is characteristic of allergic asthma is explained by the presence of a macrophage-like APC population that persists in the airway lumen [105]. Pulmonary macrophages produce a variety of factors that directly stimulate airway smooth muscle contractility and degradation of the ECM that contributes to pathological airway remodeling. Airway macrophages from some asthmatics are bathed in

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type-2-associated cytokines, including IL-4, IL-13, and IL-33, causing their differentiation that have been implicated in the pathogenesis of asthma [2]. These macrophages in turn promote the production of type-2 cytokines by pulmonary CD4 T lymphocytes and produce a variety of cytokines and chemokines that regulate the recruitment of eosinophils, Th2 cells, and basophils to the lung suggesting a viscous cycle that worsens disease[7]. Adoptive transfer studies have shown that the severity of allergen-induced disease is exacerbated by IL-4R$^+$ macrophages [106], while protection from allergic airway disease is associated with a reduction in IL-4R$^+$ macrophages in some studies [107]. Increased numbers of IL-4R$^+$ macrophages have also been reported in the lungs of asthmatic patients that have reduced lung function [108]. Nevertheless, studies conducted with LysM(cre)*IL4R*α$^{-/lox}$ mice in which Cre-mediated recombination results in deletion of the IL-4Rα chain in the myeloid cell lineage surprisingly identified no substantial role for IL-4Rα activated macrophages in ovalbumin- and house dust mite-induced allergic airway disease [109].

Macrophages have also been implicated in the pathogenesis of a variety of autoimmune diseases including rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease. In these diseases, macrophages are an important source of many of the key inflammatory cytokines that have been identified as important drivers of autoimmune inflammation, including IL12, IL18, IL23, and TNFα[110]. Macrophage-derived IL23 promotes end-stage joint autoimmune inflammation in mice. TNFα also functions as an important driver of chronic polyarthritis, while IFN-γ- and TNFα-dependent arthritis in mice has been attributed to macrophages and DCs producing IL-18 and IL-12. The pathogenesis of chronic demyelinating diseases of the central nervous system (CNS) has also been attributed to macrophages displaying a pro-inflammatory phenotype. These inflammatory macrophages contribute to axon demyelination in autoimmune encephalomyelitis (EAE) in mice, a frequently used model of multiple sclerosis. Consequently, novel therapeutic strategies that target specific myeloid cell populations could help ameliorate pathogenic inflammation in the CNS [111]. The pathogenesis of inflammatory bowel disease is also tightly regulated by inflammatory macrophages. A subset of TLR2$^+$ CCR2$^+$ CX3CR1^{int} Ly6C^{hi} Gr-1$^+$ macrophages has been shown to promote colonic inflammation by producing TNFα [112]. A recent study showed that inflammatory mediators produced in the colon convert homeostatic anti-inflammatory macrophages into proinflammatory DC-like cells that are capable of producing large quantities of IL12, IL23, iNOS, and TNF [113]. CD14$^+$ macrophages producing IL23 and TNF have also been identified in Crohn's disease patients [103]. Thus, macrophages and DCs are key producers of many of the cytokines that have been implicated in the pathogenesis of IBD.

While there is substantial evidence supporting roles for inflammatory macrophages in autoimmune inflammation, numerous studies have also reported suppressive roles for macrophages. For example, macrophages producing reactive oxygen species can protect mice from arthritis by inhibiting T cell activation [114]. Pro-inflammatory cytokines produced by activated macrophages have also been shown to protect mice from Crohn's disease by facilitating the clearance of pathogenic commensal bacteria from the mucosal lining of the bowel [115]. Recruited monocytes and resident tissue macrophages are also thought to maintain homeostasis in the intestine by clearing apoptotic cells and debris, promoting epithelial repair, antagonizing pro-inflammatory macrophages, and by producing the suppressive cytokine IL-10, which is critical for the maintenance of FoxP3 expression in colonic Treg cells [113,115,116]. Macrophages also protect rodents from demyelinating diseases of the CNS by promoting T cell apoptosis and by expressing the anti-inflammatory cytokines TGFβ1 and IL-10. The inhibitory receptor CD200 (also known as OX2), which is also expressed on antiinflammatory macrophages, has been shown to prevent the onset of EAE in mice [117]. A unique population of monocyte-derived macrophages also reduces inflammation resulting from spinal cord injury, providing further evidence of a protective

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role for macrophages in the CNS [118]. Together, these observations illustrate how changes in macrophage differentiation in the local milieu can play a decisive role in the pathogenesis of a wide variety of autoimmune and inflammatory diseases.

Macrophages in Fibrosis

Although macrophages phagocytose and clear apoptotic cells as a part of their normal homeostatic function in tissues, when they encounter invading organisms or necrotic debris following injury, they become activated by endogenous dangers signals and PAMPs. These activated macrophages produce anti-microbial mediators like reactive oxygen and nitrogen species and proteinases that help kill invading pathogens, which helps restore tissue homeostasis. However, they also produce a variety of inflammatory cytokines and chemokines like TNFα, IL1, IL6, and CCL2 that help drive inflammatory and anti-microbial responses forward [8,72], which exacerbate tissue injury and in some cases lead to aberrant wound healing and ultimately fibrosis (scarring) if the response is not adequately controlled, as has been demonstrated by the selective depletion of macrophages at various stages of the wound healing response [119]. Therefore, in recent years research has focused on elucidating the mechanisms that suppress inflammation and prevent the development of fibrosis. Although most wound healing responses are self-limiting once the tissue-damaging irritant is removed, in many chronic fibrotic diseases the irritant is either unknown or cannot be easily eliminated [120]. In this situation, it is imperative that the macrophage response converts from a pro-inflammatory phenotype to one exhibiting antiinflammatory, suppressive, or regulatory characteristics so that collateral tissue damage is kept at a minimum (Figure 4). A variety of mediators and mechanisms have been shown to regulate this conversion including the cytokines IL4 and IL13, Fcγ receptor and Toll like receptor signaling, the purine nucleoside adenosine and A2A receptor signaling, prostaglandins, Tregs, and B1 B cells[120,121], to name just a few. Each of these mediators has been shown to activate distinct populations of macrophages with suppressive or regulatory characteristics. These “regulatory” macrophages express a variety of soluble mediators, signaling intermediates, and cell surface receptors including IL-10, Arginase-1, IKK alpha, MMP13, maresins, CD200 (Ox-2), Relm-alpha, and PD-L2, which have each been shown to decrease the magnitude and/or duration of inflammatory responses, and in some cases contribute to the resolution of fibrosis [7]. They also produce a variety of soluble mediators including CSF-1, insulin-like growth factor 1, and VEGF that promote wound healing [122]. Consequently, in addition to promoting fibrosis, macrophages are also intimately involved in the recovery phase of fibrosis by inducing ECM degradation, phagocytosing apoptotic myofibroblasts, and by dampening the immune response that contributes to tissue injury [120]. Therefore, current fibrosis research is focused on characterizing these regulatory macrophage populations and devising therapeutics strategies that can exploit their anti-inflammatory, anti-fibrotic, and wound healing properties.

Perspectives

The understanding of macrophage biology is rapidly expanding with an appreciation of their diverse origins, transcriptional complexity, lability and phenotypic switching according to homeostatic demands and in response to insult. Macrophages are involved in almost every disease and thus represent extremely attractive therapeutic targets that either augment or inhibit their responses. However for these therapies to be effective it is necessary to understand macrophage diversity and define the cells according to anatomical location and function as well as the regulation of the particular set-points that defines the particular recognizable macrophages such as microglia, osteoclasts and Kuppffer cells. In fact the recognition of multiple origins (YS, FL, BM) may result in the conclusion that there is no such thing as a “macrophage” but rather clades of cells that have similar characteristics but with different origins. In fact, their different origins may allow unique opportunities to

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selectively target the recruited ones in the context of the chronic diseases discussed above thereby inhibiting the pathology without disturbance of resident macrophages and thereby normal homeostasis. To define these similarities and differences it will be necessary to determine proteomes and transcriptomes of particular subtypes as was recently performed for resident macrophages [1]. Clearly the rapidly advancing field of genomic analysis will provide unique insights and novel ways of defining macrophage types. Furthermore macrophage biology in humans is poorly developed because of the obvious technical limitations of getting fresh material for FACS and the over-reliance in functional and genomic studies on cell lines such as the myelomonocytic leukemic cell line THP1 [123] or the in vitro differentiation of circulating monocytes by CSF-1. Significant differences also exist between human and mouse macrophages for example the inability of human macrophages to increase arginase-1 expression that is an important marker of IL4-regulated macrophages in mice [3]. These differences render even binary classifications such as M1 and M2 wanting let alone more complex ones. Definition of human macrophage diversity is just beginning [124] with several ongoing sequencing efforts and these efforts will enhance the essential need to translate mouse biology into the human context.

Considerable advances in defining macrophage biology have recently been made using mouse genetic approaches. For example the use of fluorescently labeled macrophages by expressing GFP from the *Csf1r* promoter to define and in some case live image them using intravital microscopy [125]. Furthermore, the development of macrophage restricted cre recombinases for example expressed from the *LysM* or *Csf1r* promoters and the ability to ablate macrophages through the expression of the Diptheria toxin receptor that sensitized mouse cells to the toxic effect of Diptheria toxin [119] or using miRNAs to direct expression of HSV TK in macrophages has been key to defining functions. While these systems have given major insights into macrophage function caution needs to be exercised as none of the promoters are uniquely expressed in macrophages and they are also expressed in most macrophage types thereby making it difficult to discriminate the functions of sub-classes of macrophages. As we move forward specific promoters will be developed to ablate genes in particular sub-sets and more sophisticated lineage tracing to follow fates and sub-type switching will be possible through photoactivatable flours such as Dendra2 that allow single or a few cells to be tracked [125].

Therapeutic targeting of macrophages is now well underway [21,23]. Most of the therapies are targeted at pan-macrophage markers such as the CSF1R. Reagents either inhibiting the ligand, ligand binding or the tyrosine kinase activity are various stages of clinical trials in cancer, including small molecules and mAbs directed against CSF1R [21]. Other strategies in fibrosis and cancer have been to target recruitment of macrophages particularly through inhibition of inflammatory monocyte trafficking with anti-CCL2 or CCR2 antibodies. In other circumstances, the protective effects of recombinant human serum amyloid P (SAP; also known as pentraxin 2) in IPF and post-surgical scarring in patients treated for glaucoma is thought to be through the reduction of inflammation and fibrosis by inducing the production of IL-10 in regulatory macrophages [107]. Neutralization of GM-CSF using antibodies is being tested in phase II trials for multiple sclerosis and Rheumatoid arthritis [21]. In the future it seems it will be possible to exploit the inherent plasticity of macrophages to adjust their set points to control obesity by down-modulating inflammatory cytokines, to resolve fibrosis by inducing the differentiation of resolving macrophages and to treat cancer by converting macrophages from their trophic to an immunologically activated anti-tumoral state.

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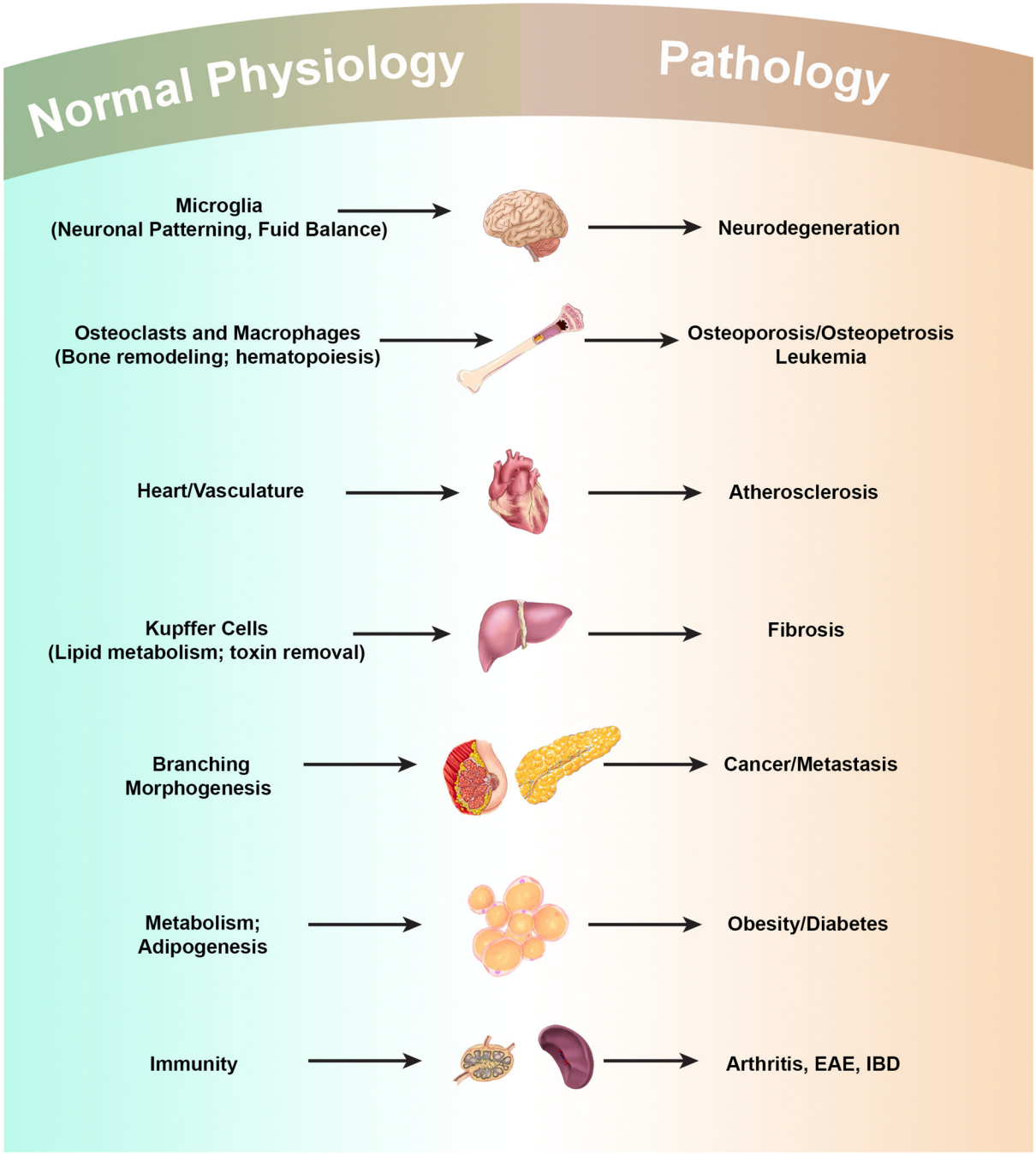

Figure 1. Macrophages in Development, Homeostasis and Disease

Macrophages play many developmental roles shaping the architecture of tissues ranging from the brain to bone to the mammary gland. Once development is over macrophages modulate homeostasis and normal physiology through regulating diverse activities including metabolism and neural connectivity and by detecting damage. These trophic and regulatory roles however, are often subverted by continuous insult and macrophages contribute to many diseases that are often associated with aging.

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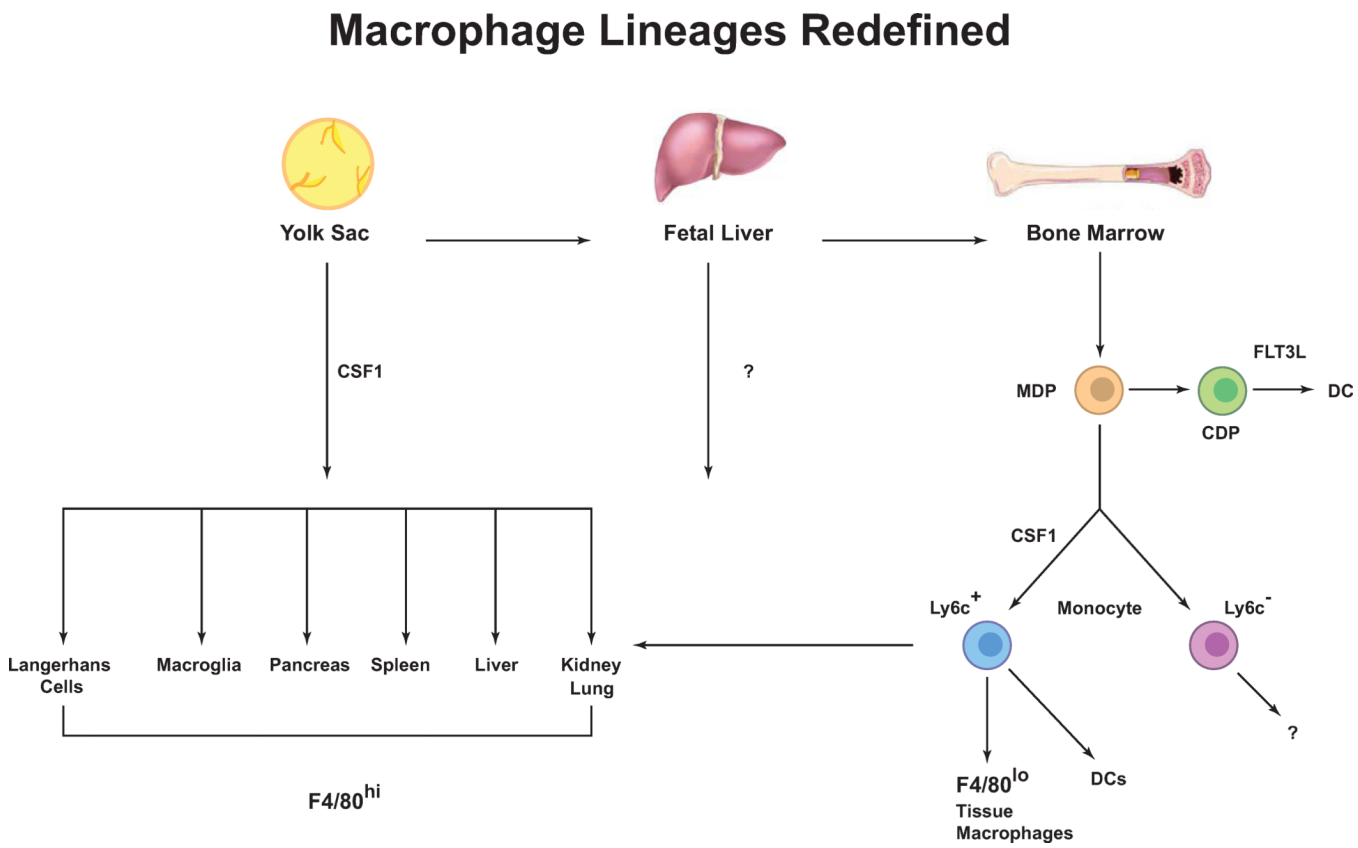

Figure 2. Macrophage Lineages Re-Defined in Mice

The mononuclear phagocytic system in adults derives from at least three sources. The first is the yolk sac (YS) that results in progenitors that populate all tissues and their progeny persist throughout life as F4/80 bright resident macrophages. These lineages are largely regulated by CSF1R. The second from the fetal liver is less well defined but seems to contribute to adult LCs perhaps through a progenitor derived from the YS. The third lineage derives from the bone marrow (BM) to give circulating monocytes and their progeny F4/80^{low} macrophages and DCs. In this case the Ly6c+ monocytes give rise to classical DCs under the regulation of FLT3 and these are continuously replenished. Other macrophages that are F4/80 low also emanate from Ly6c^{+} monocytes and in some cases such as kidney and lung, co-exist with those derived from YS to give chimeric organs. The exact role of the patrolling Ly6cmacrophages remains unclear, as is the contribution of fetal liver to adult tissue macrophages.

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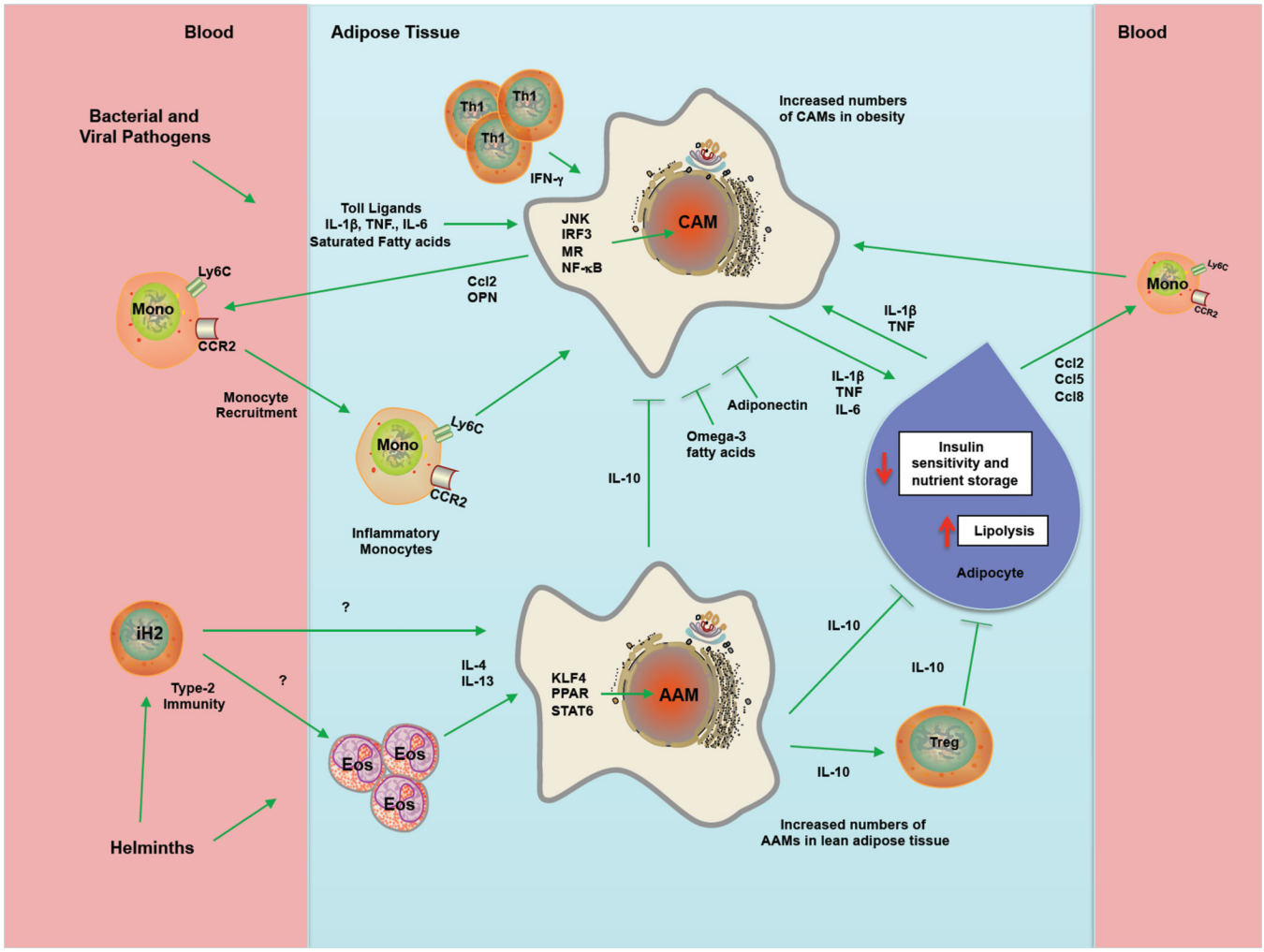

Figure 3. Activated and alternatively activated macrophages differentially regulate insulin sensitivity in obesity

In lean healthy animals, adipose tissue macrophages comprise 10–15% of stromal cells and express markers that link them with alternatively-activated macrophages, which are critical for maintaining insulin sensitivity in adipocytes, in part via the production of IL-10. Type-2 cytokines such as IL4 and IL13 derived from a variety of cellular sources, including eosinophils, appear to be important for maintaining the AAM phenotype in lean tissues. In contrast, during obesity Ly6C^{hi} monocytes are recruited, which increases macrophage content to 45–60%. These macrophages, in contrast to normal resident macrophages, express an inflammatory phenotype, characterized by the production of TNFα, IL6 and IL1β These inflammatory macrophages decrease insulin sensitivity while facilitating the storage of excess nutrients. The enlarging white adipose tissues in turn release chemokines, such as CCL2, CcCL5, and CCL8, to recruit additional Ly6C^{hi} inflammatory monocytes that further exacerbate the process. This mechanism is also enhanced during bacterial and viral infections so essential nutrients are diverted to lymphocytes, which must utilize glycolysis to enhance their activation at times of stress.

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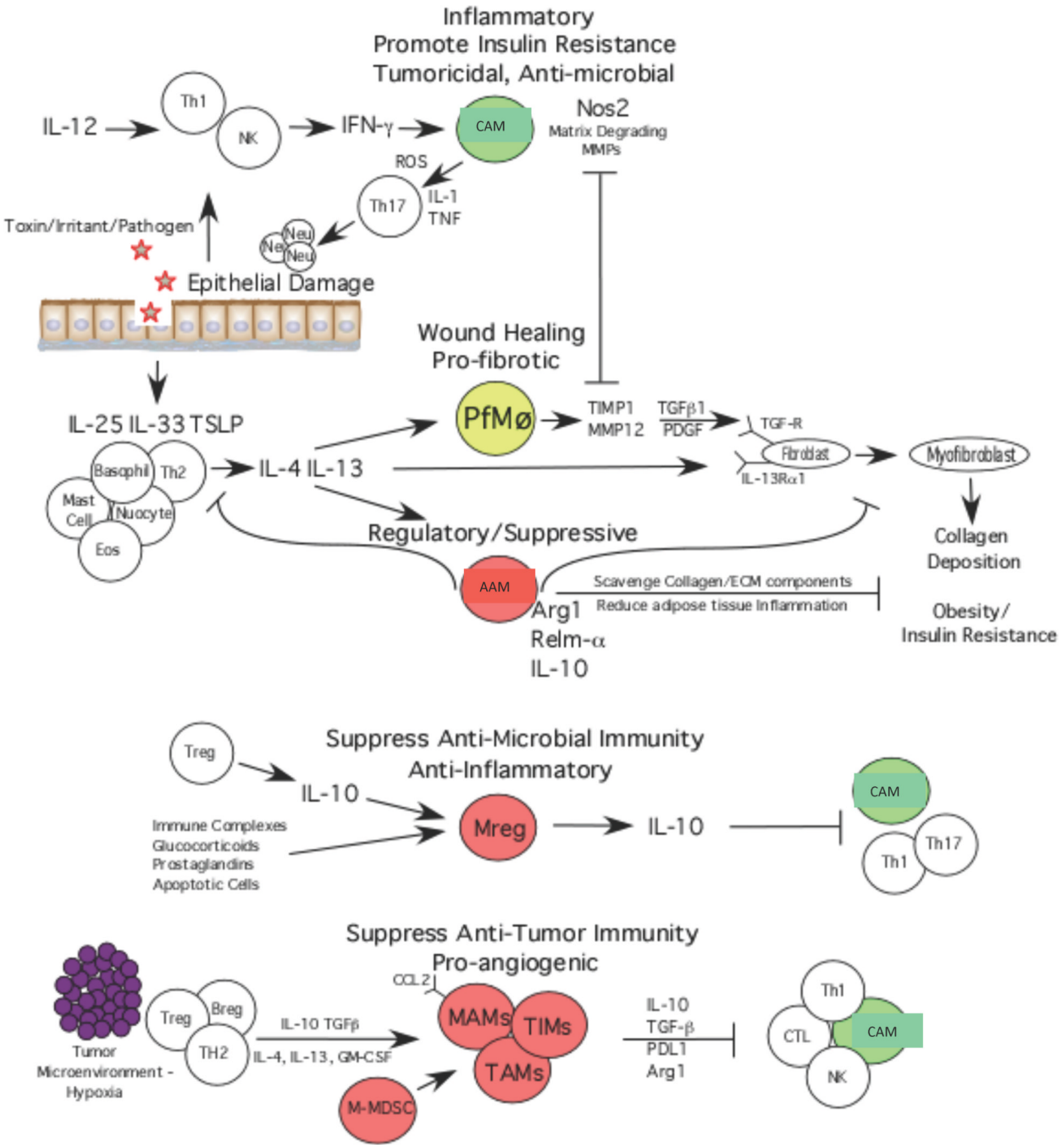

Figure 4. Macrophages exhibiting unique activation profiles regulate disease progression and resolution

Macrophages are highly plastic cells that adopt a variety of activation states in response to stimuli found in the local milieu. During pathogen invasion or following tissue injury, local tissue macrophages often adopt an activated or “inflammatory phenotype”. These cells are commonly called “classically activated” macrophages (CAM), because they were the first activated macrophage population to receive a formal definition. These macrophages are activated by IFN-γ and/or following Tolllike receptor engagement, leading to the activation of the NF-kβ and Stat1 signaling pathways, which in turn increases production of reactive oxygen and nitrogen species and pro-inflammatory cytokines like TNF-α, IL-1, and IL-6

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that enhance antimicrobial and anti-tumor immunity, but may also contribute to the development of insulin resistance and diet-induced obesity. Epithelial derived alarmins and the type-2 cytokines IL4 and IL13, in contrast, result in an “alternative” state of macrophage activation that has been associated with wound healing, fibrosis, insulin sensitivity, and immunoregulatory functions. These wound healing, pro-angiogenic, and pro-fibrotic macrophages (PfMø) express TGF-β1, PDGF, VEGF, WNT ligands, and various matrix metalloproteinases that regulate myofibroblast activation and deposition of extracellular matrix components. Alternatively activated macrophages (AAMs) also express a variety of immunoregulatory proteins like arginase-1 (*Arg1*), Relm-alpha (*Retnla*), *Pdl2*, and *Il10* that regulate the magnitude and duration of immune responses. Therefore, in contrast to CAMs that activate immune defenses, AAMs are typically involved in the suppression of immunity and re-establishment of homeostasis. Although type-2 cytokines are important inducers of suppressive or immunoregulatory macrophages, it is now clear that several additional mechanisms can also contribute to the activation of macrophages with immunoregulatory activity. Indeed, IL10-producing Tregs, Fc gamma receptor engagement, engulfment of apoptotic cells, and prostaglandins have also been shown to preferentially increase the numbers of regulatory macrophages (Mregs) that suppress inflammation and inhibit anti-microbial and anti-tumor defenses. The tumor microenvironment itself also promotes the recruitment and activation of immune inhibitory cells, including those of the mononuclear phagocytic series such as myeloid-derived suppressor cells (MDSCs), tumor-infiltrating macrophages (TIMs), tumor-associated macrophages (TAMs) and metastasis-associated macrophages (MAMs) that promote angiogenesis and tumor growth, while suppressing anti-tumor immunity.