[Frontiers in Bioscience 14, 4079-4102, January 1, 2009]

Survival of monocytes and macrophages and their role in health and disease

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1. ABSTRACT

Macrophages are versatile cells involved in health and disease. These cells act as scavengers to rid the body of apoptotic and senescent cells and debris through their phagocytic function. Although this is a primary function of these cells, macrophages play vital roles in inflammation and repair of damaged tissue. Macrophages secrete a large number of cytokines, chemokines and growth factors that recruit and activate a variety of cell types to inflamed tissue compartments. These cells are also critical in cell-mediated immunity and in the resolution of inflammation. Since macrophages, and their precursors, blood monocytes, are important in regulating and resolving inflammation, prolonged cellular survival in tissue compartments could be detrimental. Thus, factors that regulate the fate of monocyte and macrophage survival are important in cellular homeostasis. In this article, we will explore stimuli and the intracellular pathways important in regulating macrophage survival and implication in human disease.

2. INTRODUCTION

Human bone marrow produces approximately $5x10^9$ monocytes per day (1). They are derived from a common myeloid precursor cell which gives rise to neutrophils and monocytes (2,3,4). However, monocytes diverge after the colony-forming unit (CFU)-granulocyte-macrophage (GM) stage to form a monoblast responsive to GM-colony-stimulating factor (CSF) and then a promonocyte which is responsive to macrophage (M)-CSF resulting in monocyte formation (Figure 1).

The sequential order between M-CSF and GM-CSF is not entirely clear. The development of transgenic mouse models suggests that M-CSF has an important function proximal to GM-CSF in the development of monocytes due to the reduction of monocyte and macrophage numbers in M-CSF deficient mice (5) compared to normal numbers of monocytes and macrophages found in GM-CSF deficient mice (3,6,4). Interestingly, mice lacking both M-CSF and GM-CSF have reduced numbers of monocytes and macrophages that are similar to M-CSF deficient mice, suggesting that M-CSF provides monocyte and macrophage survival and differentiation effects (7). In addition, M-CSF deficient animals largely correct their endogenous monocyte and macrophage deficiency over the life-span of these animals and an important factor in this replenishment of mononuclear phagocytes is interleukin-3 [IL-3] (8).

In the peripheral blood, monocytes circulate as innate immune surveillance cells. Upon chemokine secretion, tissue injury, or pathogen signal, monocytes are recruited to the affected area and diapedese across the blood vessel into the inflamed tissue (9,1,10). Once in the tissue, the monocyte encounters a wide variety of proteins, including cytokines, matrix proteins, and growth factors that instruct the cell to complete their differentiation program. Recent work demonstrates that there is heterogeneity among circulating monocyte populations based on surface markers and that these cells selectively respond to different inflammatory conditions (9,10). In the absence of inflammation, the monocyte circulates for a short span of 24-48 hours and either becomes a tissue macrophage or dies apoptotically (1). In contrast to a monocyte, the life span of a macrophage dramatically increases to months to years (11).

The combination of the tissue microenvironment, including matrix components, growth factors and other cells, defines the outcome of the macrophage in the tissue. Interferon-gamma (IFN-γ) released by activated T-lymphocytes and endotoxin at sites of inflammation enhances production of "classical" or M1 phenotype macrophages (12). M1 macrophages produce Th1 cytokines (IL-12) and express inducible nitric oxide synthase [iNOS] (13,14). M2 macrophages are alternatively activated by TH2 cytokines and express arginase. Functionally, M1 macrophages are responsible for anti-microbial responses and tissue damage, while M2 macrophages promote allergic responses and facilitate tissue remodeling and repair (13).

In the inflammatory milieu, macrophages have multiple functions, including acting as phagocytic cells to clear the area of cell debris and remodeling and repair of damaged tissue (15,13). These cells produce matrix metalloproteinases (MMPs) to remodel the tissue matrix and instruct other cells to deposit collagen and other basement membrane proteins essential for repair and remodeling.

Circulating monocytes give rise to tissue macrophages that have diverse organ locations including Kupffer cells in the liver, alveolar macrophages in the lung, microglial cells in the brain, peritoneal macrophages, osteoclasts in the bone, and mammary macrophages in the breast (16,13) [Figure 1]. In addition to these tissue cells, monocytes can also differentiate into dendritic cells. In the laboratory, dendritic cells can be derived from bone marrow in the presence of GM-CSF, Flt3-ligand (Flt3-L) or IL-3 (17). However, combining these cytokines with IL-4, IL-13, transforming growth factor (TGF)-β, or interferon (IFN)-β enhances dendritic cell production (18,17). It is likely that the combination of cytokines stimulate monocytic subpopulations to differentiate to dendritic cells (10,17).

Resident macrophages not only have phagocytic functions, but also have additional regulatory roles, including host defense, homeostasis and cellular communication (16,19,20). The regulation of tissue macrophages is controversial. Many feel that resident macrophages are descendents of pre-hematopoietic precursor cells that migrate to their perspective tissues during embryogenesis (13,20,21,22). It is recognized that tissue macrophages can proliferate in the tissue environment and self-renew (13). Additionally, studies comparing cell surface marker expression profiles between circulating monocytes and resident macrophages in mice reveal subtle cell surface marker homology, suggesting that a subpopulation of monocytes may populate tissue macrophage stores (20,23,24,19). In fact, Kupffer cells are repopulated from blood monocytes in the adult (22), although the subpopulation of monocytes in humans is not well delineated. It is likely that this occurs for all other tissue macrophage populations in the body. In bone marrow transplant patients, lung macrophages transition to donor phenotype (25) suggesting a common bone marrow source of these cells. These observations are true for lung, liver and microglial macrophages in mouse transplant models (23,24). However, when the irradiation regimen is altered in mice to selectively damage bone marrow cells but not the resident lung macrophages then the lung macrophages remain that of the host (26). Thus, it is apparent that multiple mechanisms are responsible for maintaining adequate tissue macrophage numbers due to turnover.

In addition to regulating maturation signals, the tissue microenvironment also influences macrophage responses. Some of these responses are beneficial, while others are detrimental. We and others found that macrophages are important angiogenic switches in tumor microenvironments (27,28,29). Eubank *et al* reported that

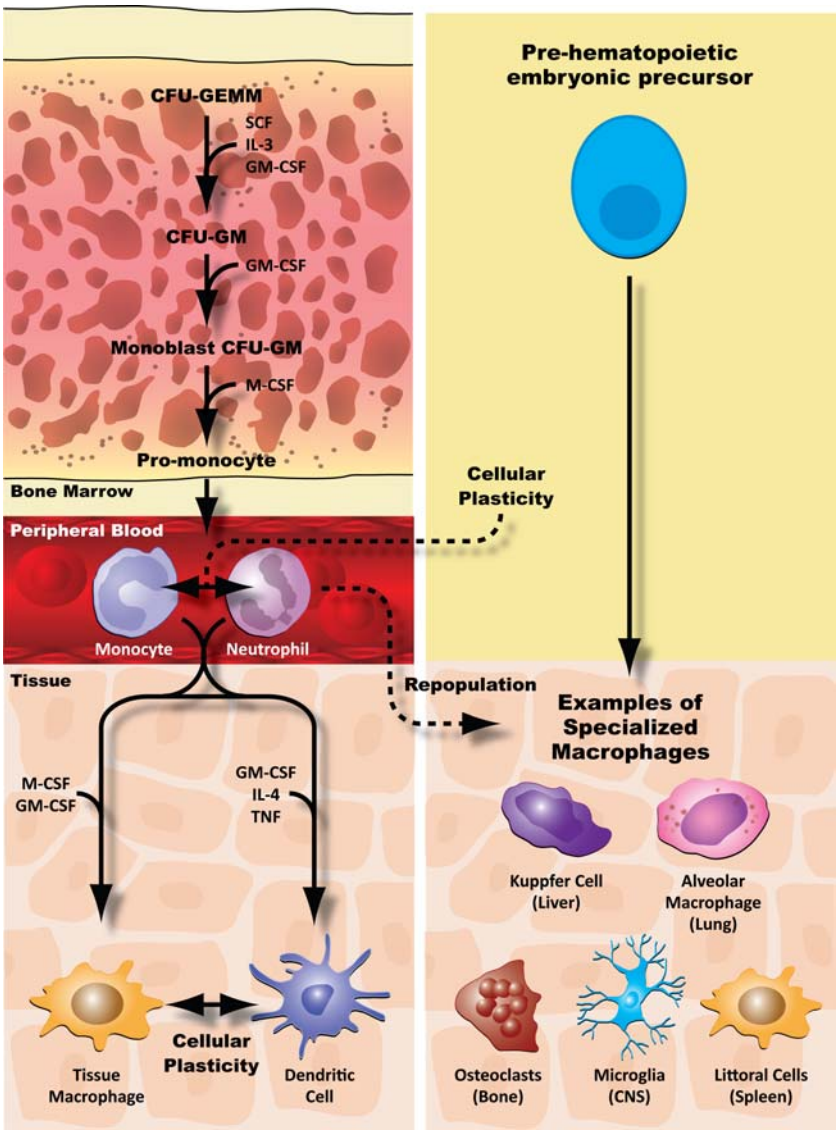

Figure 1. Macrophage development. Macrophages are derived early in embryogenesis prior to the development of a functional bone marrow environment. However, the exact cells responsible for the development of fetal macrophages have not been clearly identified but most likely would originate from the fetal liver. Once the bone marrow takes over hematopoiesis, monocytes are produced in response to cytokines and released in the peripheral blood. Once in tissues, the monocytes differentiate to tissue macrophages. The microenvironment of the tissue dictates the type of specialized macrophage that will be formed with unique functions compared to other tissue macrophages. Notably, circulating monocytes can repopulate the specialized macrophages. Recently, cellular plasticity between specific macrophage types as well as neutrophils becoming macrophages has been reported. Although, it is possible that this phenomena involves circulating stem cells or other immature cells.

while macrophages treated with M-CSF or GM-CSF secrete VEGF, cells treated with GM-CSF also secrete the soluble form of VEGF receptor-1, which sequesters VEGF, blocking endothelial cell proliferation and angiogenesis (29). It is possible that tumors exploit tumor-associated macrophages to produce blood vessels leading to tumor growth and metastases. Re-educating these macrophages in the tumor environment may allow manipulation of these signals and improvement in the outcome of the disease.

Until recently, inflammatory cells like macrophages and neutrophils were thought to be terminally differentiated. Surprisingly, Hume and colleagues demonstrated that activated mouse neutrophils express the M-CSF receptor and differentiate into macrophages *in vitro* after M-CSF treatment (30). Interestingly, neutrophils express mRNA for M-CSF receptor but not the protein. The protein is expressed only after overnight incubation in culture. Hume's observations is not entirely new, as others showed that human neutrophils treated with a variety of

cytokines including GM-CSF and M-CSF assume a macrophage phenotype (31).

It is also has been reported that macrophages can transdifferentiate. In particular, certain macrophage subsets like dendritic cells can transdifferentiate to other macrophage subsets including osteoclasts (32,33). Previous work demonstrates that myofibroblasts (34) and proliferating smooth muscle cells also express M-CSF receptors (35), suggesting the possibility of a common precursor or transdifferentiation of these cells to an alternate phenotype. Notably, macrophages treated with pleotrophin become functional endothelial cells (36), suggesting another role in which macrophages can contribute to wound healing and revascularization of tissue.

Understanding how growth factors like M-CSF influence macrophage survival in areas of acute inflammation is critical to clarify mechanisms of chronic inflammation. The data reviewed above raises important questions about the transition of a neutrophilic infiltrate to one predominated by macrophages during the transition from acute to chronic inflammation and in the resolution of inflammation. However, since M-CSF and its receptor are important in macrophage production and are associated with the genesis of numerous diseases, the majority of this review will center on M-CSF in regulating macrophage homeostasis and the role of both M-CSF and macrophages in human disease.

3. CYTOKINES AND NON-CYTOKINES THAT ACTIVATE MONOCYTE/MACROPHAGE SURVIVAL

3.1. GM-CSF and IL-3 as monocyte survival factors

Once monocytes enter inflamed tissue, growth factors such as M-CSF or GM-CSF drives monocyte differentiation into macrophages. Interestingly, macrophage numbers are reduced in mice lacking M-CSF (5) but not in mice lacking GM-CSF (6). The loss of GM-CSF renders macrophages defective in phagocytic capacity and maturation (6). In addition to M-CSF and GM-CSF, IL-3 also activates survival pathways in blood monocytes and facilitates macrophage differentiation (4,7).

Gene knockout studies in mice suggest the biological role for GM-CSF and IL-3 as "emergency" responders during immune challenge and inflammation as opposed to maintaining homeostatic levels of granulocytes and macrophages (7,6). Notably, mice deficient in either of these cytokines have normal myeloid cell numbers but are susceptible to infections. During an inflammatory insult, pro-inflammatory cytokines including TNF-α, IL-2, IL-1, and IFN-γ induce endothelial cells and fibroblasts to secrete GM-CSF which in return induces myelopoiesis from the bone marrow (4).

Normally, the receptor machinery for GM-CSF and IL-3 signaling is expressed on most types of myeloid progenitor cells, macrophages, granulocytes, and dendritic cells (37). On human cells, each GM-CSF and IL-3 receptor has a specific, cognate receptor subunit for binding ligand, GM-CSFα and IL-3α, respectively. After ligand binding, these low-affinity binding subunits form ternary complexes with the high-affinity common-β (βc) subunit and transduce signaling events to the nucleus. While humans express a single βc subunit that is shared among GM-CSF, IL-3, and IL-5, mice express a shared common βc receptor for GM-CSF and IL-5 and an exclusive βc for the IL-3 receptor (4). In humans, the pattern and abundance of common-β receptor expression on certain cell populations in local environments govern responsiveness to either GM-CSF or IL-3 (38).

There are distinct regions of the intracellular domains on the βc receptor that regulate cell differentiation, proliferation, or survival. For example, the membrane proximal 35 amino acids are essential to stimulate a mitogenic response, but this domain alone is unable to support cell survival (3). Some of the same survival pathways activated by the M-CSF receptor are also triggered by GM-CSF and IL-3. Upon ligand binding to the α receptor subunits for GM-CSF or IL-3, the βc becomes tyrosine phosphorylated. Since the βc lacks intrinsic kinase activity, it relies on the kinase activity of JAK2 to become phosphorylated leading to the activation of signaling intermediates such as phosphatidylinositide 3 (PI3)-kinase, Ras/MAPK; phosphatases such as SH2-containing phosphatase-2 (SHP2) and SH2-containing inositol phosphatase (SHIP); and transcription factors such as signal transducer and activator of transcription-5 (STAT5) (37). Signaling events mediated by JAK2 and the Src family kinases appear to be important in cell cycle regulation and survival. Induction of negative feedback molecules including suppressor of cytokine signaling (SOCS) which downregulates JAK2 signaling also occurs.

Notably, there is no known binding site on the βc receptor for the p85 subunit of PI3-kinase which activates Akt signaling and cell survival (39). However, certain adaptor proteins can associate with βc receptor resulting in the recruitment of either p85 or products downstream of PI3-kinase (37).

GM-CSF induces gene expression profiles similar to M-CSF during differentiation. Hashimoto et al used serial analysis of gene expression, or SAGE, to measure gene regulation of either M-CSF- or GM-CSF-induced cellular differentiation (40). Interestingly, GM-CSF-stimulated macrophages are round in appearance while M-CSF-stimulated macrophages are spindle-shaped (1,41). Despite this phenotypic difference in cell appearance, the majority of genes expressed in GM-CSF- versus M-CSF-induced macrophages are similar (40). Of 35,037 different transcripts identified, genes expressed in highest number during monocyte to macrophage differentiation were involved in lipid metabolism. When comparing M-CSF to GM-CSF, 117 transcribed genes of GM-CSF- and M-CSF-induced macrophages are expressed at significantly different levels. Of the 117 transcribed genes, 57 are increased in GM-CSF-treated macrophages compared with M-CSF-treated macrophages, and the remainder are elevated in M-CSF-treated macrophages (40).

3.2. ROS/LPS

Free radicals are molecules containing one or more unpaired electrons and include reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS

include hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals, whereas RNS include nitric oxide and peroxynitrite (42,43). Intracellular ROS are generated through a variety of sources including the mitochondria electron transport chain, cytochrome p450, NADPH oxidase complex, lipoxygenase pathway, cyclooxygenase pathway, xanthine oxidase complex and peroxisomes (44). Extracellular ROS are generated through oxidative stress, such as ultraviolet (UV) or ionizing irradiation and some chemical agents (45). We demonstrated that human monocytes produce ROS after M-CSF stimulation and these endogenous ROS play important roles in monocyte survival (46,47).

The production of ROS can be harmful to cells through the oxidation of proteins and/or nucleotides resulting in single strand DNA breaks (48). However, ROS may be beneficial in limiting inflammation. Milla *et al* showed that myeloperoxidase (MPO)-derived oxidants reduce lung inflammation by increasing macrophage and T-lymphocyte apoptosis, as well as reducing pro-inflammatory cytokine and chemokine production (49). ROS regulate cellular proliferation and differentiation through the activation of intracellular signaling cascades and transcription factors such as NF-κB (50,51,45). ROS also are important in promoting angiogenesis after VEGF stimulation (52).

Although ROS can promote cell death (53,54,55), intracellular ROS can also induce cell signaling and survival (56,57). Importantly, the cellular effects of ROS are dependent on the location, type and concentration of ROS and the phase of the cell cycle (57). We found that inhibition of M-CSF-induced ROS production in monocytes using n-acetyl cysteine or the flavoprotein inhibitor diphyeleneiodonium increase Annexin V and propidium iodide (Annexin V/PI) staining indicating that endogenous ROS are important in cell survival (58). We further examined the intracellular pathways involved in M-CSF/ROS-mediated survival in monocytes/macrophages. We found that the NADPH oxidase complex I is important in generating the M-CSF-mediated ROS and activating the serine/threonine kinase Akt1. We also found that extracellular-regulated kinase (Erk) and p38 mitogen-activated protein kinase (MAPK) are activated to promote cellular survival in M-CSF-stimulated mononuclear phagocytes and are both dependent on ROS production (46,58). Importantly, Erk activation is not mediated by the NADPH oxidase complex in M-CSF-stimulated monocytes, as NADPH oxidase $p47^{phox}$ deficient macrophages activate Erk normally in response to M-CSF (58). We are actively investigating the source of ROS in mediating Erk activation.

As mentioned above endotoxin or lipopolysaccharide (LPS), a component of the cell membrane of Gram-negative bacteria promotes maturation and activation of macrophages by its interaction with the toll-like receptor 4 (59). This alternative activation of monocytes results in the development of a M2 macrophage (60). These cells are furthered classified as M2a, M2b, and M2c macrophages (60). M2a macrophages are activated by Th2 cytokines (IL-4 and IL-13) and produce the anti-inflammatory cytokine IL-10. The M2b subset of macrophages responds to LPS or IL-1β through the IL-1 receptor or the TL4 receptor and immune complexes through Fc receptors. These cells also secrete high levels of IL-10 and low levels of IL-12 to modulate a TH2 immune response and promote IgG1 antibody production. Notably the M2c macrophages respond to IL-10 as well as TGFβ or glucocorticoids. In addition, bacterial components, also activate NF-κB this leads to upregulation of Bcl-2 family members to promote cell survival (61,62,63) and will be discussed in greater detail below.

3.3 Expression and function of the M-CSF receptor

M-CSF is a disulfide-linked homodimer, which regulates cellular events through activating the tyrosine kinase M-CSF receptor (also called CSF-1R or c-fms) (64,65). The M-CSF receptor is encoded by the proto-oncogene *c-fms* (*CSFR1*). This receptor contains an extracellular ligand binding, five Ig-like domains, a short transmembrane domain, an intracellular kinase domain and a kinase insert domain (Figure 2). The binding of M-CSF to the receptor induces receptor dimerization and autophosphorylation of five tyrosine residues [Figure 2] (64). The phosphotyrosine residues serve as docking sites for adaptor and signaling molecules. Receptor activation induces a cascade of signaling proteins culminating in cell differentiation, proliferation, and survival (66,64,67,68,58,16) .

Until recently, M-CSF receptor expression was thought to be limited to cells of monocytic origin. As mentioned, recent studies show that neutrophils express mRNA for the M-CSF receptor (30) and the cells can be induced to express the surface protein. M-CSF stimulation of cells expressing M-CSF receptors promotes a macrophage phenotype, suggesting the possibility that the coordinated expression of growth factor receptors may direct early responder neutrophils to transdifferentiate into macrophages. In addition to neutrophils, other cells like osteoclasts, proliferating smooth muscle cells, myofibroblasts and endothelial cells also express M-CSF receptors (69,34,70,35). This new concept may lead to better understanding of inflammatory disease.

4. SIGNALING PATHWAYS ACTIVATED BY M-CSF TO REGULATE CELL SURVIVAL

4.1. Role of Akt in monocyte/macrophage survival

The PI3K/Akt pathway plays an important role in promoting survival of normal to transformed cells (39). PI3K consists of p110 catalytic subunit and a p85 regulatory subunit. In human monocytes, activation of M-CSF receptor by survival stimuli results in the binding of the p85 subunit to the receptor. The p85 subunit becomes phosphorylated and recruits the p110 subunit to the plasma membrane (39). Subsequently, PI3K catalyzes the transfer of a phosphate to the inositol ring of the phosphoinositides, to generate phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol 3,4,5 trisphosphate ($PI(3,4,5)P_3$). The generation of these phospholipids in the membrane leads to Akt recruitment via pleckstrin

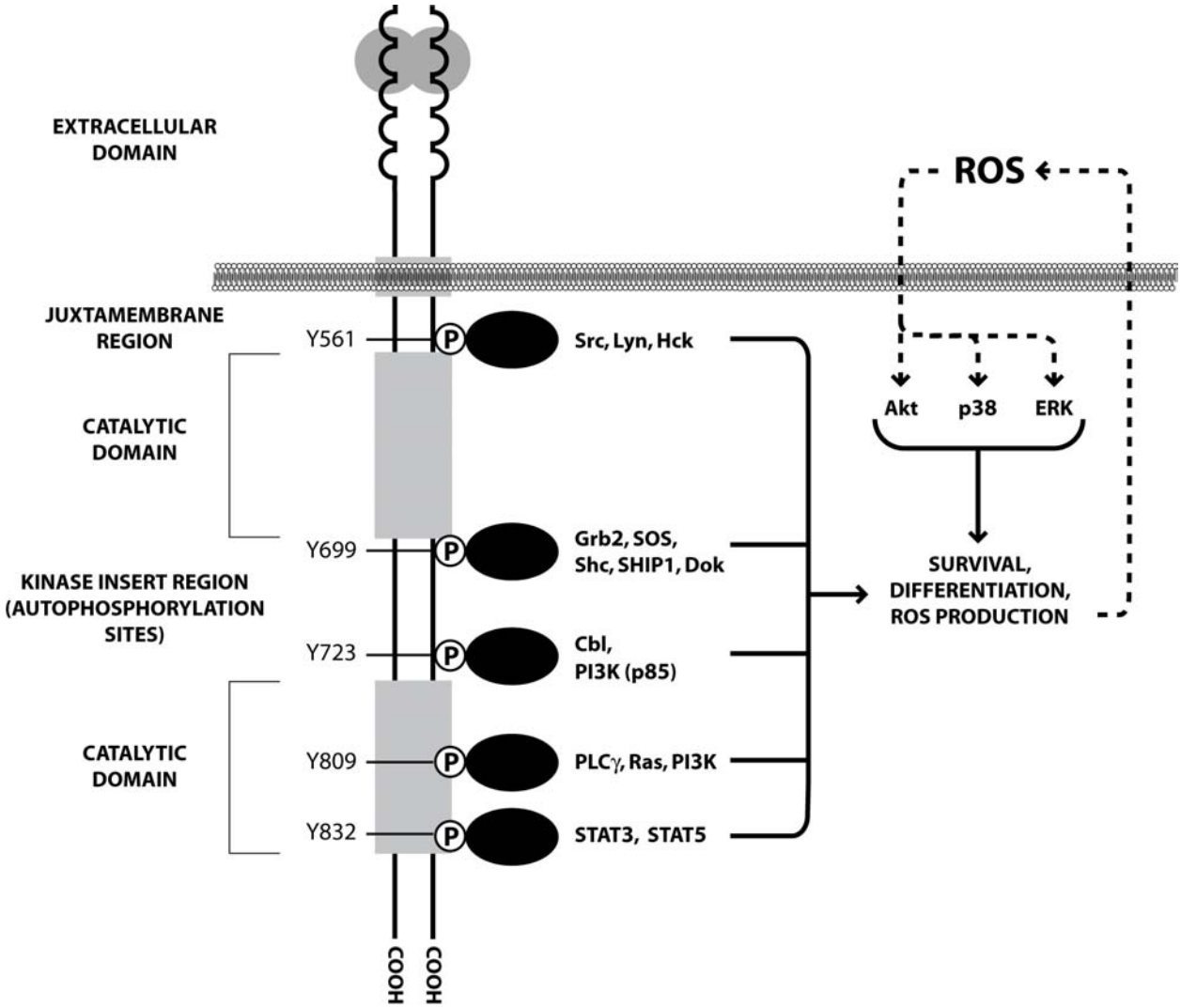

Figure 2. Structure and signaling cascades of the M-CSF receptor. The M-CSF receptor comprises of an extracellular domain that binds ligand resulting in receptor dimerization. Once dimerized the intracellular catalytic domains mediate the autophosphorylation of five tyrosine residues located at amino acid residues 561, 699, 723, 809, and 832. These tyrosine residues serve as docking sites for numerous signaling molecules many which cumulate in cell differentiation and/or cell survival. Activation of the MAPK which is downstream of Shc, Grb2, and SOS can regulate the production of reactive oxygen species (ROS). Our laboratory found that ROS can also influence macrophage cell survival through the activation of Akt, p38 and Erk.

homology (PH) domains. Akt binding to membrane-bound phosphoinositides targets Akt and induces a conformational change in Akt, rendering the regulatory subunit accessible to 3'-phosphoinositide-dependent protein kinases (PDKs). PDK1 is constitutively activated and phosphorylates Akt at Thr^{308}, promoting phosphorylation of Akt on the carboxyl terminus at Ser^{473} and activation of the kinase (71). While some reports suggest that the Ser^{473} phosphorylation of Akt is mediated by autophosphorylation, others report PDK2/MapKK (72), PKC-β2 (73) or integrin-linked kinase (ILK) are responsible (74). For full activation and biological functions of Akt, Src mediated tyrosine phosphorylation at Tyr^{315} and Tyr^{326} appears to be important (75). Members of the Src kinase family are also activated by M-CSF and will be discussed below.

Akt promotes cell survival by stimulating survival proteins like Bcl-x_L, Bcl-2 and reducing expression of apoptotic proteins such as Bax and BAD, apoptosis signal kinase-1 (ASK-1) and caspase-9 (76,62). Furthermore, Akt regulates several transcription factors including NF-κB, E2F, forkhead transcription factors, CREB, Yes-associated protein (YAP) (76), as well as the cell cycle regulator p21 (77).

Of the three known mammalian isoforms of Akt (Akt1, Akt2, and Akt3), Akt1 is most commonly reported to augment cellular survival (76). In human peripheral blood monocytes, growth factors such as M-CSF and GM-CSF; bacterial cell wall products like LPS; and inflammatory cytokines such as IL-1β, tumor necrosis

factor (TNF)-α, and IL-18 promote monocyte survival by inducing Akt1 phosphorylation and kinase activity (66,76,11,58). The PI3K inhibitors LY294002 and wortmannin block cytokine-induced monocyte survival by suppressing Akt1 activation and increasing caspase-3 and caspase-9 activity (67,11). Notably, blocking caspase-9 activity using the LEHD-fmk inhibitor only partially reverses cell apoptosis indicating that multiple pathways are involved in regulating macrophage cell survival (67). Despite overlapping signaling pathways to support monocyte survival, several studies suggest that Akt is a principle survival factor. Over expression of activated Akt1 enhances human peripheral monocyte and bone marrow-derived macrophage survival (58). In addition, expression of a catalytically inactive Akt1 (K179M) along with stable expression of the human M-CSF receptor in NIH 3T3 fibroblasts results in apoptosis even in the presence of M-CSF (67). Moreover, SHIP1 and phosphatase and tensin homolog (PTEN) knockout mice (78,67,79) demonstrate enhanced Akt1 activation and suffer organ-based inflammation. More details of these phosphatases will be discussed under "Models of enhanced macrophage trafficking" (*Section 7.2)*.

4.2. Role of MAPK family members in monocyte/macrophage survival

MAPKs activate intracellular pathways in response to growth factors, stress, and inflammatory cytokines (80). There are three major subfamilies of MAPKs; Erk (81,82), p38 MAPK, the c-Jun NH_2–terminal kinase (JNK) (83,80,84). A fourth subfamily may also exist (80). Generally, the Erk members respond to growth factors, reactive oxygen species and phorbol esters, while p38 MAPK and JNK members are more sensitive to stress stimuli (81,82).

There are several layers of complexity in the signaling pathways generated by MAPKs. MAPKs are regulated by a series of consecutive phosphorylation events (83,80,84). A significant amount of data is known for the pathways leading to the activation of the "classic Erk kinases", Erk1 and Erk2, which are the downstream targets in the Ras/MAPK pathway and activated by hematopoietic growth factors, including M-CSF (85).

We and others found that M-CSF stimulates Erk1/2 activation in human PBMs as well as *ex vivo*-derived mouse bone marrow macrophages (46,64). M-CSF-mediated Erk1/2 activation is dependent on the phosphorylation of Tyr^{809} in the M-CSF receptor. At this residue, components assemble that activate Ras and Raf-1 leading to the phosphorylation of MEK1/2 upstream of Erk1/2 (80). This pathway is also important in M-CSF-induced cellular differentiation (86).

Several Ras-independent mechanisms exist to activate MAPK. Protein kinase C (PKC) members which are activated by M-CSF can also activate these proteins (87,88). PKC proteins phosphorylate the Raf kinase inhibitor protein to release Raf-1 and phosphorylate MEK (87). We recently reported that exogenous ROS can induce Erk activation in human monocytes in the absence of M-CSF, but do not independently support monocyte survival (58). In contrast, endogenous ROS generated after M-CSF stimulation of macrophages regulates Erk1/2 activation and monocyte survival (46).

Erk plays an important role in monocyte homeostasis, survival and differentiation after growth factor receptor stimulation (86). Inhibition of Erk leads to monocyte apoptosis (46). After M-CSF stimulation, PI3K inhibitors suppress M-CSF-induced Erk activation in human monocytes, suggesting that PI3K products are upstream of Erk activation (46). PI3K inhibitors block M-CSF-induced ROS, perhaps by interrupting Akt or Rac1 (58). Following activation, Erk promotes the activation of transcription factors like Ets-2 and NF-κB (63,89). The PI3K/Akt and the MAPK pathways, regulate cell survival at different levels. While the MAPK regulates key components at a transcriptional level, PI3K/Akt targets proteins at a post-transcriptional level (58). Based on the stimuli one or both pathways may regulate macrophage survival.

Unlike the ability of catalytically active Akt expression to promote cell survival (58), expression of constitutively activated Erk does not lead to the same outcome (90). Reddy and coworkers reported that phagocytosis of apoptotic bodies by macrophages promoted survival in the absent of serum or survival factors through the activation of Akt and concomitant inhibition of Erk1/2 (90). Unlike apoptotic cells, necrotic cells activate, rather than inhibit, Erk1/2 activity. These signaling differences may explain the opposing tendencies between apoptotic cells and danger signals such as LPS, TNF-α, IFN-γ and bacterial DNA in promoting an inflammatory response (91,92).

In mammalian cells, p38 MAPK responds to environmental stress, inflammatory cytokines, chemokines, or LPS, but does not respond to mitogenic stimuli (80,93). p38 MAPK is critical in normal and inflammatory responses in macrophages, neutrophils and T-cells by affecting respiratory burst, chemotaxis, endocytosis, adherence, and apoptosis of these cells (93).

Like other cells, the involvement of p38 MAPK in monocyte/macrophage cellular survival or apoptosis is cell and environment specific. For example, cardiomyocytes or fibroblasts lacking p38 MAPK are resistant to apoptosis (94). In contrast, p38 MAPK activation protects neuronal PC12 cells from TNF-α-induced apoptosis and enhances osteoblastic SaOS-2 cell growth. We found that ROS-mediated p38 MAPK activation contributes to the survival of primary human monocytes (58). Other investigators reported that p38 MAPK play no role on monocytic cell line survival (95). Thus, it appears that cell type and stimulus powerfully influence the effect of p38 MAPK on cell life or cell death.

Similar to p38, the JNK pathway is activated by cytokines and stress signals, like DNA-damaging agents, UV irradiation, and serum deprivation. These stress signals activate transcription factors including c-Jun, ATF2, NF-ATc1, STAT3, and HSF-1 (81,96). Similar to p38 MAPK

pathways, the involvement of JNK in cell survival and apoptosis are diverse (94). A recent study shows that JNK pathway inhibition increases macrophage apoptosis following exposure to shiga toxin (Stx1) in THP-1 cells, while p38 MAPK inhibition reduces cell killing (95). On the contrary, other investigators demonstrate that JNK is important in macrophage survival and development (97). JNK inhibition results in cell cycle arrest at the G_2/M transition, causing apoptosis. JNK inhibition also downregulates M-CSF receptor (c-fms) and Bcl-x_L mRNA expression in mature macrophages and represses M-CSF-dependent differentiation of bone marrow cells to macrophages (97). In fact, JNK inhibition disrupts the binding of the transcription factor, PU.1, to promoter elements found in these genes.

4.3. Other signaling pathways activated by M-CSF

The Src families of kinases are implicated in multiple signaling pathways that regulate cellular growth, migration, differentiation and survival. Src family kinases contain nine members: Blk, Fyn, Fgr, Hck, Lck, Src, Yes and Yrk. Among those kinases, Hck, Lyn and Fgr are restricted to myeloid cells and B lymphocytes (98). Src family kinases contain an amino terminal cell membrane anchor and in its inactive state folds back upon itself to inhibit its catalytic activity (99).

Src family kinases are both positive and negative modulators of cellular signaling by growth factors, including M-CSF. Following M-CSF stimulation, Src, Fyn, and Yes become activated (100,101). Mutational analysis of the M-CSF receptor demonstrates that all three of these kinases can associate with the M-CSF receptor at Tyr^{559} (100). Mutation of this tyrosine residue is associated with reduced receptor autophosphorylation. However, expression of this receptor isoform leads to a hyperproliferative phenotype due to reduction in receptor ubiquitination resulting in high levels of receptor surface expression.

Src family kinases also interact with Tyr^{809} within the M-CSF receptor (100,101). During macrophage differentiation, Fyn binds to the M-CSF receptor and becomes activated (102). This interaction is dependent on protein kinase C (PKC) activity.

The role of Src family kinases in regulating monocyte survival has been reported. In M-CSF-stimulated monocytic THP-1 cells exposed to bacterial cell wall products, expression of Hck is induced to modulate cell survival (103). Our laboratory reported that Lyn negatively regulates M-CSF-induced monocyte survival by recruiting SHIP1 to the cell membrane and down regulating PI3K/Akt activation (66). Moreover, *Lyn*$^{-/-}$ macrophages have increased Akt1 activity in response to M-CSF.

STAT proteins also interact with the M-CSF receptor (104,105,5). Based on consensus sequences, there are several potential residues in the M-CSF receptor where STATs could interact. Following their activation, STAT proteins form homo- and heterodimers and then translocate to the nucleus to transactivate promoter elements (37). To date, the role of STATs in mediating M-CSF-induced cell survival is not known. However, since STATs transactivate Bcl-x_L and Bcl-2, it is likely that they promote cell survival (37).

5. TRANSCRIPTION FACTORS DOWNSTREAM OF M-CSF ACTIVATION

5.1. Role of NF-κB in monocyte/macrophage survival

The NF-κB family of transcription factors is composed of several structurally-related DNA-binding proteins that recognize a common sequence motif (106,107). In resting cells, NF-κB is sequestered in the cytoplasm by a family of IκB inhibitory proteins masking the nuclear localization signal. Following treatment of cells with various cytokines and growth factors, including M-CSF (108), NF-κB translocates to the nucleus and induces gene transcription (109). NF-κB is present in most cells, including mononuclear phagocytes, and the majority of proteins encoded by NF-κB target genes are pro-inflammation and pro-survival including Bcl-x_L, X-IAP, IAP-1 and -2, Iex-1_L, Mcl-1, and Bfl-1 (110,111).

NF-κB induces macrophage survival and maintains mitochondrial homeostasis (61). We previously reported that SHIP1 and SHIP2, negative regulators of M-CSF-induced Akt activation, suppress NF-κB activity in M-CSF-stimulated cells (66,68). We are completing work on the mechanism used by M-CSF to regulate NF-κB activity in monocyte survival and differentiation.

5.2. Ets transcription factors

The Ets transcription factors regulate proliferation, differentiation, cell survival, and cell migration and contain more than 30 members. Their functions are regulated through the highly conserved amino acid motif (ETS domain) that recognize and bind the core recognition DNA sequence, GGAA/T (112). Ets2 is ubiquitously expressed and is a target of the MAPK kinase pathway (112,113). In macrophages, M-CSF induces Ras/Erk-dependent phosphorylation of Ets2 at Thr^{72} leading to induction of Ets2/M-CSF target genes; urokinase plasminogen activator (uPA), Bcl-x_L, scavenger receptor A, MMP-1 and MMP-9, TNF-α, IL-1β, and chemokines, CCL2/MCP-1 and CCL3/MIP-1α (89,114). M-CSF-induced activation of PI3K/Akt also regulates Ets2 phosphorylation (114) providing a powerful link between Ras/Erk and Akt in regulating the survival of mononuclear phagocytes.

Recently, Wei *et al* showed that the inflammatory phenotype of SHP-1 is largely regulated by the activation of the transcription factor Ets2 (115). This murine model has been useful in understanding lung disease and will be discussed later.

6. PHOSPHATASES THAT REGULATE MONOCYTE/MACROPHAGE SURVIVAL

6.1. SHIP1

SHIP1 is a 145 kDa protein and is primarily expressed in hematopoietic cells and becomes tyrosine

phosphorylated upon growth factor stimulation (116). SHIP1 hydrolyzes the 5′ phosphate from the second messengers phosphatidylinositol (PI) (1,3,4,5)P_4 and PI(3,4,5)P_3, converting them to PI(1,3,4)P_3 and PI(3,4)P_2, limiting Akt membrane localization and activation (116,117,118,119).

SHIP1 contains an N-terminal SH2 domain, a central catalytic core, and a C-terminal domain containing two NPxY motifs. After cytokine stimulation, SHIP1 becomes phosphorylated and binds Shc and other signaling proteins, including Grb2 (120). Membrane targeting of SHIP1 is critical for its function as a negative regulator of cell proliferation and survival. SHIP1 also reduces mast cell degranulation (121) and cytokine-induced neutrophil survival (122,123).

We reported that SHIP1 negatively regulates M-CSF-induced signaling in monocytes (66). SHIP1 binds to the Src-family kinase Lyn at the membrane to negatively regulate Akt activation in M-CSF-stimulated cells. Our observations are relevant as SHIP1 plays a physiological role in human diseases characterized by enhanced cellular survival. In chronic myelogenous leukemia (CML), the *BCR/ABL* oncogene suppresses the expression of SHIP1, suggesting that SHIP1 contributes to the pathogenesis of CML (124). Importantly, forced expression of SHIP1 or re-expression of SHIP1 using STI-571 in CML cells leads to their death (124,125).

6.2. SHIP2

Similar to SHIP1, SHIP2 is a 5′-inositol phosphatase. However, while SHIP1 expression is limited to hematopoietic cells, SHIP2 is ubiquitously expressed (119,126,127). SHIP2 also interacts with Shc but has additional distinct binding partners after cytokine stimulation. For example, whereas SHIP1 binds Grb2 and Src, SHIP2 binds abelson tyrosine kinase (Abl) (119). Another defining characteristic between SHIP1 and SHIP2 is in the inability of SHIP2 to hydrolyze PI(1,3,4,5)P_4, however like SHIP1, SHIP2 negatively regulates PI(3,4,5)P_3 (128).

While SHIP2 is a close structural and functional homologue of SHIP1, SHIP2 is transcribed as an independent gene and encodes a protein with a predicted molecular mass of 142 kDa (129). The C-terminal region of SHIP2 is distinct from SHIP1. In this region, lies only one NPxY motif that becomes phosphorylated upon stimulation with various stimuli, including insulin and M-CSF (130,127,68,131,132).

SHIP2 has been extensively studied in insulin signaling. SHIP2 predominantly regulates Akt2 phosphorylation at the plasma membrane in response to insulin in 3T3-L1 adipocytes (133). Polymorphisms in SHIP2 are associated with metabolic syndromes including type 2 diabetes and hypertension (134,135). Recently, we found that SHIP2 down-regulates FcγR-mediated phagocytosis, independent of SHIP1 (136). We also found that SHIP2 negatively regulates M-CSF-induced signaling via the regulation of Akt and NF-κB activity (137,68).

6.3. PTEN

PTEN is another negative regulator of the PI3K pathway. This lipid phosphatase dephosphorylates the 3′ position of PI(3,4,5)P_3, reducing the second messenger required for Akt activation (138,139,140). Unlike the SHIP proteins, mutations reducing PTEN function occur in a wide variety of cancers and thus PTEN is considered a tumor suppressor (141,142). As expected, *PTEN* $^{-/-}$ mice have elevated levels of Akt1 phosphorylation and activity and decreased apoptosis. They die during embryogenesis from failure of developmental apoptosis (143,144,139). The double transgenic *PTEN*$^{+/-}$ *Akt1*$^{-/-}$ mice have normal life spans and Akt deficiency suppresses tumor development in these mice (145).

The regulation of PTEN activity occurs at transcriptional, translational, and post-translational levels. Various transcription factors including p53 (146) and Egr (147) increase PTEN expression. In contrast, NF-κB activation reduces PTEN expression and prevents apoptosis in human lung and thyroid cancer cells (148). Interestingly, human alveolar macrophages have reduced PTEN levels and elevated Akt activity (58). Reduction in PTEN levels correlates with increased basal ROS levels in macrophages (149). Since PI3K is important in regulating NAPDH oxidase complex activity and regulates Rac1, these data suggest that PTEN regulates PI3K-dependent ROS in phagocytic cells.

6.4. SHP-1

SHP-1 (known as SH-PTP1, PTP1C and HCP) is a 65kD protein tyrosine phosphatase expressed predominantly in hematopoietic cells (150,151,152,150). Molecular and structural protein analysis reveals that SHP-1 contains two N-terminal SH2 domains, a phosphatase domain and two tyrosine phosphorylation sites in the C-terminal region of the protein. The phosphatase activity of SHP-1 is regulated by intramolecular interactions, such that the N-terminal SH2 domain is folded over the phosphatase domain in the inactive state (153,154,154). The enzyme becomes activated when the amino-terminal SH2 domain is engaged by a phosphopeptide, which allows the phosphatase domain to access its substrate. Thus SHP-1 activity is upregulated by addition of a cognate phosphopeptide or by deletion of the N-terminal SH2 domain. SHP-1 negatively regulates a wide variety of growth factor receptors including the M-CSF receptor (155), epidermal growth factor receptor (156), and c-kit receptor (157,158,157).

In normal macrophages stimulated with M-CSF, SHP-1 becomes tyrosine phosphorylated (159,160). Despite not directly binding to the M-CSF receptor, SHP-1 influences M-CSF receptor phosphorylation by directly inactivating signaling proteins. Following M-CSF stimulation, SHP-1 forms complexes with Cbl, STAT3, STAT5a,/b, PI3K, Shc, vimentin and Grb2 resulting in their dephosphorylation (155,160). In murine macrophages, SHP-1 constitutively associates with the Ras-GAP-associated protein, $p62^{DOK}$ and upon its phosphorylation undergoes a conformational change to expose its catalytic domain and dephosphorylate $p62^{DOK}$ (161).

The functional role of SHP-1 as a negative regulator of signaling emerged with the identification of mice with naturally occurring mutations in the SHP-1 gene. Notably, these mice are the naturally occurring model of pulmonary fibrosis (162) and will be described in greater detail in *Section 7.2*.

7. MURINE MODELS OF DISEASE RELATED TO MONONUCLEAR PHAGOCYTE BIOLOGY

7.1. Murine models deficient in macrophages

7.1.1. M-CSF$^{-/-}$ and M-CSF receptor null models

Understanding the function of M-CSF has been enhanced by the identification of osteopetrotic (op/op) mice. These mice harbor an inactivating mutation within the gene for M-CSF (*Csf1*) (5). As mentioned previously, the homozygous mice (*Csf1^{op}/Csf1^{op}*) have severe reduction in tissue and resident macrophages numbers contributing to osteopetrosis, reduced weight, impaired neuronal development, infertility and lack of teeth. Additionally, these mice have shortened life-spans due to a compromised immune system. Daily injections of M-CSF starting on the third day after birth partially or completely reverse many defects (163). To fully determine if the lack of M-CSF is responsible for the phenotype of the *Csf1^{op}/Csf1^{op}* mouse, Ryan and colleagues re-introduced the full length CSF-1 gene in the *Csf1^{op}/Csf1^{op}* mouse (163,164). Re-expression of the M-CSF gene corrects all defects in the osteopetrotic mice.

Not surprising, mice with a targeted disruption in the c-fms gene encoding the M-CSF receptor also develop osteopetrosis (165,166). *Csf1r$^{-/-}$* mice are nearly undistinguishable from *Csf1^{op}/Csf1^{op}* mice, however this mutation increases the reduction of tissue macrophages. One important observation is that these mice have significant elevations in serum M-CSF levels. Since *Csfr$^{-/-}$* mice with increased amounts of circulating M-CSF have similar phenotypes to M-CSF deficient mice, it is likely that M-CSF does not bind to secondary receptors.

Since M-CSF deficient mice are protected from vascular diseases like atherosclerosis and transplant vascular disease (167,168,169,170), we utilized *Csf1^{op}/Csf1^{op}* mice to understand macrophage contribution in lung fibrosis. We found that *Csf1^{op}/Csf1^{op}* mice have less lung inflammation and fibrosis when challenged with intraperitoneal bleomycin than wild type mice. In addition, the absence of M-CSF decreases expression of CCL2, CCL12, and CTGF. Similarly, CCL2 deficient animals are protected from intraperitoneally-injected bleomycin-induced lung injury. Collectively, we found reduced numbers of cells recruited to the lungs after treatment in both models suggesting that M-CSF mediates tissue repair and remodeling by recruiting inflammatory cells and fibrocytes through upregulation of chemokines.

7.1.2. PU.1 deficient mice

PU.1 transcription factor is a member of the Ets domain-containing transcription factor family (171). PU.1 is similar to the Spi-B transcription factor forming a subfamily within the Ets family. PU.1 is highly expressed in myeloid cells and B-cells. Expression of PU.1 is tightly regulated during hematopoietic development. Several PU.1 gene targets in mononuclear phagocytes are the M-CSF receptor, GM-CSF receptor and CD11b.

The absence of PU.1 is embryonic lethal due to the of lack B-cells, neutrophils and macrophages (172). However, these mice are also deficient in T-cells demonstrating the role of PU.1 in myeloid and lymphoid lineage commitment. A second PU.1 knockout mouse is viable if maintained with antibiotic therapy (173). This mouse lacks monocytes, neutrophils and B-cells but has T-cells. As expected, resident liver macrophages are absent. *In vitro* differentiation of embryonic stem cells from PU.1 deficient mice fail to generate macrophages indicating the importance of PU.1 and M-CSF receptor expression in macrophage differentiation (173)

7.2. Models of enhanced macrophage trafficking

7.2.1. SHIP deficient mouse models

SHIP1 deficient mice are viable, but have a shorter life-span and are smaller than wild type littermates (78). The mice have splenomegaly, hyper-hematopoiesis and accumulation and infiltration of granulocytes and macrophages in the lung due to defects in executing apoptosis and excessive cellular survival in myeloid lineages. Bone marrow-derived macrophages from *SHIP1$^{-/-}$* mice are responsive to M-CSF and show prolonged Akt1 phosphorylation after M-CSF stimulation (174,78).

Unlike SHIP1, SHIP2 plays an important role in physiology. With the initial generation of SHIP2 knockout mice, investigators found early mortality due to insulin hypersensitivity, severe neonatal hypoglycemia and deregulated expression of the genes involved in gluconeogenesis (175). Our laboratory found that *SHIP2$^{-/-}$* fetal liver-derived macrophages have augmented M-CSF-induced Akt1 activity (68). However, unlike macrophages from SHIP1-deficient mice which display constitutively active Akt1, basal Akt1 activity is reduced in SHIP2-deficient cells.

During the generation of the initial SHIP2 knockout mice, both SHIP2 and Phox2a genes were inadvertently deleted (176). Another SHIP2 knockout mouse has been generated and survives to adulthood. Unlike the original SHIP2 deficient mice, these mice have no defects in insulin and glucose homeostasis. Notably, when fed a normal diet, the serum triglycerides, non-esterified free fatty acid cholesterol and leptin levels are lower than wild type mice despite similar metabolic rates (176,177). These mice are also highly resistant to weight gain on high fat diets, exhibiting an obesity-resistant phenotype. It will be of utmost interest to examine the function of M-CSF on macrophages from this mouse model.

7.2.2. Myr-Akt1 mouse model

Akt1 is an important macrophage survival factor, thus generation of a mouse expressing a membrane-targeted form of Akt1 in monocytic lineage cells was predicted to be informative. The myristoylated (Myr)-Akt1

mouse was generated using the c-fms promoter to direct expression in mononuclear phagocytes (178). Since active Akt requires membrane localization, the myristoylation tag facilitates membrane binding resulting in constitutive activation of Akt1.

Based on the SHIP1 mouse model, one would predict macrophage accumulation in these mice. However we and others have not seen this effect in non-challenged mice (unpublished data). However, the basal activation of Akt1 in bone marrow-derived macrophages is increased (58) and others have reported the activation of downstream targets of the Akt signaling cascade under resting conditions in these macrophages (178). In fact, Myr-Akt1 macrophages survive better than wild type cells after withdrawal of M-CSF (58). This observation mimics the result of expressing Myr-Akt1 in human peripheral blood monocytes.

In addition to alterations in survival, Myr-Akt1 mice display aberrant phagocytosis of bacteria and have enhanced production of IL-10 when challenged with LPS. Bone marrow-derived macrophages display enhanced FcγR-mediated phagocytosis (178). These data underscore the importance of PI3K/Akt and the downstream target NF-κB in the production of pro- and anti-inflammatory cytokines from LPS-treated human monocytes (179).

7.3. Murine models of human disease

7.3.1. Pulmonary fibrosis

The functional role of SHP-1 as a negative regulator of growth factor signaling emerged with the identification of mice with point mutations in the SHP-1 gene (180,181). There are two predominant mutations resulting in either the expression of a catalytically inactive splice variant or a complete loss of SHP-1 protein expression. The animals bearing the former mutations are designated as motheaten viable (Mev), and those bearing the latter mutation are designated as motheaten (Me) (182,183,184,181,182,182,181). Importantly, these mice have been reported as a naturally occurring model of pulmonary fibrosis (162). Both of these SHP-1 mutations result in a severe phenotype, with uncontrolled expansion of myeloid cells in a number of organs, including the lung resulting in a shortened life-span of about 5-10 weeks. Specifically, T and B cells from SHP-1 deficient animals are hyper-responsive to immune receptor stimulation (185,186,187,188,189,186,190,189,191,188,192). In motheaten mice lacking active SHP-1 but not wild type mice, the M-CSF receptor is hyperphosphorylated implying that SHP-1 regulates M-CSF receptor phosphorylation events (155).

More recently, Wei *et al* reported that the inflammatory phenotype of SHP-1 motheaten mice was largely due to enhanced activation of the transcription factor Ets2 (115). When transgenic animals expressing a mutated form of Ets2 (*Ets2*^{T72A}) are crossed with the motheaten animals, the inflammatory motheaten phenotype is corrected. Since M-CSF activates Ets2 and Ets2 upregulates survival proteins, it is possible that this pathway may be important in the development of lung inflammation and fibrosis.

7.3.2. Atherosclerosis

Atherosclerosis is an inflammatory disease with intimate involvement of macrophages (193,194). The role of macrophages during the formation of an atherosclerotic plaque is initially passive as lipid-laden macrophages form the foundation of the fatty streak (193). The release of oxidized low density lipoproteins (LDL) induces the recruitment of monocytes to the vessel wall. Continual exposure of cytokines such as M-CSF enables ongoing recruitment and survival of macrophages releasing matrix metalloproteinases, chemokines and cytokines to recruit additional cells and induce the rupture of the plaque, thrombosis, and ischemia (193,194). Additionally, M-CSF mediates the uptake of LDL and acetylated LDL by macrophages to transform them to foam cells (195). Interestingly, intimal smooth muscle cells (SMC) also express the M-CSF receptor (35) suggesting both a pathological mechanism by which M-CSF release in the plaque could mediate plaque rupture and the possibility that plaque macrophages or influxing monocytes may transdifferentiate into smooth muscle cells.

Mice deficient in apolipoprotein E (ApoE) are hypercholesterolemic and develop atherosclerosis on a low fat diet (170). When ApoE deficient mice are bred with *Csf1*^{op}*/Csf1*^{op} mice, the resultant progeny (Op0/E0) have significantly less atherosclerosis than wild type mice, despite elevated plasma cholesterol levels compared to littermate controls (195,170,196). Importantly, expression of one wild type copy of the M-CSF gene in the ApoE deficient mouse decreases cholesterol levels but returns atherosclerotic lesions to that seen in wild type mice (195).

Analysis of the macrophages in the Op0/E0 mice found lesions containing less foam cells and reduced numbers of macrophages in coronary arteries and aortas (197,196). Scavenger receptor (SR-A) expression is responsible for cholesterol uptake and is reduced proportionally to macrophage numbers in these mice. While reduced in number, the receptor is functional and mediates catabolism of acetylated LDL in these mice. Notably, defective clearance of β-VLDL by macrophages contributes to increased plasma cholesterol levels. These studies demonstrate the importance of M-CSF-induced monocyte/macrophage activation in regulating cholesterol levels and development of atherosclerosis.

Monocyte recruitment to the atherosclerotic lesion is also important in the inflammatory response in coronary artery disease. Hypercholesterolemia induces CCL2 expression in SMC and upregulates expression of its receptor CCR2 on monocytes (198). Local CCL2 expression results in monocyte recruitment following vascular injury. Mice lacking CCL2 and LDL receptor also have less atherosclerosis when fed diets rich in cholesterol (199).

Notably, when *ApoE*$^{-/-}$ mice are fed a high fat diet, increased CCL2 expression appears within one week

(198). Within 24 hours of vascular injury in these mice, CCL2 is enhanced in SMC and in platelets adherent to the vessel wall. In fact, anti-CCL2 therapy in ApoE deficient mice inhibits the formation of atherosclerosis (200).

Similar to *ApoE*$^{-/-}$ mice, *ApoE/CCR2* double knockout mice are hypercholesterolemic (201). As expected, atherosclerosis is attenuated in these mice when fed a high fat diet. *ApoE*$^{-/-}$/*CCR2*$^{-/-}$ mice are also protected following vascular injury and have reduced neointimal lesions with a significant decrease in recruited macrophages (198).

Fractalkine (CX3CL1) chemokine is expressed both as a soluble and a membrane bound form. Expression of CXC3L1 has been detected in endothelial cells as well as macrophages in atherosclerotic lesions (202). Monocytes and a subset of lymphocytes migrate in response to soluble CX3CL1 due to expression of the receptor CX3CR1. Mice deficient in both CXC3R1 and ApoE also have reduced atherosclerosis (203).

Lastly, *ApoE*$^{-/-}$ mice treated with angiostatin have reduced angiogenesis and fewer macrophages (204). Macrophages regulate angiogenesis in an M-CSF-dependent fashion through VEGF production (27,29), enabling additional inflammatory cell recruitment to the lesion. Collectively, these studies demonstrate the importance of monocyte recruitment and survival in coronary artery disease.

7.3.3. Breast cancer

Clinically, there are many reports linking tumor-associated macrophages with poor prognosis. Macrophages promote tumor angiogenesis by secreting a milieu of growth factors, cytokines, chemokines and proteolytic enzymes which provide a favorable environment for vascular invasion. In humans, M-CSF expression, as well as expression of its cognate receptor c-fms, corresponds to high grade disease and poor prognosis (205). Expression of M-CSF is also prevalent in invasive tumor cells as opposed to pre-invasive phenotypes (28)

Transgenic mouse models of breast cancers that mimic the stages of human breast cancer phenotypically and molecularly have been generated. Muller *et al* found that mice expressing another oncoprotein, the polyoma virus middle-T antigen (PyMT), associates with and activates tyrosine kinase activity of a number of c-Src kinase family members (Src, Yes, and Fyn). PyMT also interacts with the p85 subunit of PI3K which is required for transformation (206). Driven by the mouse mammary tumor virus (MMTV) promoter and expressed specifically in the mammary glands, PyMT overexpression induces multifocal adenocarcinomas in the mammary glands of mice.

M-CSF overexpression in mice leads to dense leukocyte infiltration within breast tumors, the majority of these cells being macrophages (207). To understand the role of mononuclear phagocyte biology in breast cancer, Lin *et al* generated transgenic mice which expressed PyMT solely in the mammary glands in combination with a recessive null mutation in the M-CSF gene. The absence of M-CSF reduces macrophage infiltration into the tumors and decreases pulmonary metastasis. Likewise, transgenic expression of M-CSF in the mammary glands of M-CSF deficient mice restores the metastatic potential of these tumors (205).

Macrophages contribute to tumor progression by altering the microenvironment through expression of pro-angiogenic factors like VEGF (27) and members of the epidermal growth factor (EGF) family (208). To understand paracrine effects that macrophages contribute to tumor progression, Ahmed *et al* generated mice expressing green fluorescent protein (GFP)-expressing carcinoma cells (driven by MMTV and co-expressing PyMT) in primary mammary tumors to allow direct examination by intravital imaging (209,210). *In vivo* chemotaxis assays showed both M-CSF and EGF gradients are required for carcinoma cells and macrophages to migrate and mediate metastases. This is interesting because macrophages express M-CSF receptors and tumor cells express EGF receptors, yet the absence of either M-CSF or EGF inhibits migration of both cell types (210).

8. ABERRANT M-CSF EXPRESSION AND/OR RECEPTOR ACTIVATION IN HUMAN DISEASE AND ITS INFLUENCE ON CELL SURVIVAL

8.1. Idiopathic pulmonary fibrosis (IPF)

IPF is an interstitial lung disease associated with abnormal tissue remodeling following lung injury, leading to collagen deposition (211). Patients with this disease have a mean survival of 3-5 years following diagnosis and currently there is no effective treatment. We recently found increased levels of M-CSF in bronchoalveolar (BAL) fluid from patients with IPF (212). We also found an association with increase CCL2 production in these patients, similar to our observations with murine models of IPF. Thus, our observations suggest that lung cells respond to elevated M-CSF levels by secreting CCL2, CTGF, and CCL12 into the lung. Increases in CCL2 and CCL12 promote fibrocyte recruitment to the lung (213,212) and CTGF expression enhances collagen expression by lung fibroblasts and myofibroblasts. These data suggest that M-CSF may recruit mononuclear phagocytes to the lung directly and through CCL2 secretion, as well as recruit fibrocytes to the lung via CCL2 and CCL12 and induce these cells to produce collagen through CTGF expression.

8.2. Lung cancer

Several investigators report increased production of CSFs in primary lung cancers (214,215). In fact, CSFs are associated with tumor progression, metastases and invasion; however, the mechanisms responsible are not elucidated (216). *In vitro*, some lung cancer cell lines produce the cytokines, G-CSF and M-CSF, and express cell surface receptors for CSFs (217). Both *in vitro* and *in vivo* studies suggest the biological effects of M-CSF on lung tumorigenesis are complex. M-CSF promotes lung cancer cell invasion via stimulation of several extracellular matrix degrading proteinases (218). However, murine xenograft

flank models demonstrated that both M-CSF gene transduction and administration of M-CSF in non-small cell lung cancer (NSCLC) slow tumor growth (219,220). Interestingly, M-CSF reduces liver and lymphatic, but not renal metastases, suggesting dependence on the tumor microenvironment (220). However, there is great variability in CSF levels in patients with NSCLC. For example, Mroczko *et al* demonstrated elevated G-CSF levels in 61 patients with NSCLC, and reduced M-CSF levels compared to normal controls (217). More recently, investigators determined in a cohort of 103 patients with previously untreated NSCLC that elevated serum M-CSF levels correlate with cancer stage and independently predict survival. There was no correlation with either tumor size or histology (221). While such findings are encouraging, the biological role for M-CSF in human lung cancer progression and its potential as a biomarker for disease remain unclear and will require further study.

8.3. Breast cancers

M-CSF is essential in normal breast development (222) and plays a role during pregnancy and lactation (223). However, expression of M-CSF and/or its receptor correlate with poor prognosis. Several studies report a significant elevation of both plasma M-CSF and the tumor marker, CA 15-3 in patients with advanced tumor stage (224). The presence of high levels of M-CSF also downregulates monocyte class II antigen expression (225) and may decrease macrophage-mediated tumor cytotoxicity and favor immune tolerance. Thus, plasma M-CSF levels in diagnosis and monitoring of breast cancer chemotherapy may serve as a marker of disease.

In vitro, c-fms-expressing BT20 human breast cancer cells have enhanced motility and invasiveness upon treatment with recombinant M-CSF (226). This model has also been used to determine the role of M-CSF-induced activation of Ets2 in tumorigenesis. Expression of dominant negative Ets2 in the BT20 cell line completely abolishes M-CSF-stimulated invasion. Furthermore, the Ets2 mutation inhibits M-CSF-induced c-myc, c-fos, and c-jun expression. These data demonstrate the importance of Ets-2 in the regulation of cellular invasiveness of transformed mammary epithelial cells.

Several mechanisms exist for the M-CSF regulation of metastases. As previously mentioned, both M-CSF and GM-CSF induce VEGF secretion from macrophages, however only GM-CSF induces the production of sVEGF to limit the activity of VEGF (29). Additionally, M-CSF can induce the expression of urokinase-type plasminogen activator (uPA) (227), a serine protease involved in extracellular matrix degradation. The breakdown of surrounding basement membrane is essential for the exposure of migratory cancer cells to the vasculature. Yee *et al* investigated the expression of M-CSF, c-fms, and uPA from human breast cancer cell lines characterized for invasive and metastatic ability. More invasive cell lines produce higher levels of M-CSF and uPA further supporting a key role for M-CSF in tumor metastases (228).

As previously discussed M-CSF plays an essential role in cellular recruitment. Thus, in the tumor microenvironment, expression of CCL2 correlates with increased macrophage recruitment into breast tumors (229). Scholl *et al* assessed M-CSF expression and the prevalence of tumor-infiltrating lymphocytes and monocytes in human breast tumors using immunohistochemistry and *in situ* hybridization from 196 breast cancer patients (230). Elevated numbers of T- and B-cell infiltrates were found in 13% and 17% of the tissue specimens, respectively. Macrophage markers, M-CSF receptors and CD68, were detected in 48% and 90% of the tumors, respectively. In addition, M-CSF was detected in 74% of the breast tumors. Notably, tumors with high percentages of M-CSF expressing cells have higher monocyte infiltrates. The presence of T-cell infiltrates and increase expression of M-CSF in the tumor was associated with the tumor metastases and poor survival.

Not surprisingly, that the clinical observations lead many investigators to target M-CSF or its receptor in mouse models of breast cancer. Targeting either M-CSF or *c-fms* mRNA using siRNA inhibits breast cancer xenografts in mice by decreasing tumor vascularity, reducing angiogenic factors and matrix metalloproteinases, and decreasing macrophage recruitment to the tumors. It will be of utmost interest to determine whether this strategy will be beneficial in humans.

8.4. Coronary heart disease

As described, there are numerous mouse models exploring the role of macrophages in the development of atherosclerosis. Blanc-Brude and colleagues reported that inhibitor of apoptosis protein (IAP) survivin is increased in atherosclerotic streaks (231). Despite high levels of other anti-apoptotic proteins, survivin levels were decreased in advance atherosclerotic plaques thus contributing to plaque instability.

Several studies examined serum levels of M-CSF in patients with coronary artery disease. M-CSF along with C-reactive protein and IL-3 are biomarkers for individuals for with chronic stable coronary heart disease (232). Patients with hemodialysis (HD) develop accelerated atherosclerosis with frequent aortic involvement compared to healthy individuals (233). HD patients have a significant increase in serum levels of M-CSF which positively correlates with aortic calcification. Of note, no association between M-CSF serum levels and cholesterol levels are observed in this patient population.

8.5. M-CSFR mutation in megakaryocytic leukemia

Until recently, no mutations have been detected in the genes for M-CSF or its receptor in human diseases. Gu *et al* are the first to report a chromosomal translocation involving the *CSFR1* (*c-fms*) gene in a human acute megakaryocytic leukemia (AMKL) cell line (234). The t(3;5)(p21;q33) translocation encodes a protein that contains the amino terminal 36 amino acids of the RNA-binding motif 6 (RBM6) protein fused in-frame to the carboxy-terminal 399 amino acids of the M-CSF receptor. The fusion protein maintains the entire intracellular domain

of the M-CSF receptor. Expression of this fusion protein in cell lines results in its constitutive phosphorylation as well as STAT5 activation leading to growth factor-independent growth. In fact, removal of the amino-terminus of the full-length M-CSF receptor also renders it constitutively active. Expression of this fusion protein in mouse bone marrow and transplanted to a receiptant mouse leads to a myeloproliferative disease. However, the incidence of this translocation in the human population is yet to be determined.

9. DISCUSSION

The importance of macrophages in the innate immune response has greatly changed the perception of their function. Much of the understanding of macrophage survival and functions has come from murine models. However, some mouse models have generated more questions than answers in the mechanism underlying macrophage maturation. What is clear from these studies is that there are factors that compensate for the loss of M-CSF, GM-CSF, or IL-3. Using mouse models to interfere with intracellular signaling cascades has been useful in determining potential therapeutic targets for human diseases. In fact, some are models for human disease such as tyrosine phosphatase SHP-1 mouse model. Other murine models help understand the important role of macrophages and M-CSF in breast cancer and atherosclerosis.

Several human diseases have an underlying problem with prolonged survival and targeting of macrophages. In some cases, increases in M-CSF circulating levels are associated with disease and are a poor prognostic factor. M-CSF affects not only cell survival but also cell recruitment, suggesting that targeting this important growth factor in human diseases may be beneficial.

10. ACKNOWLEGDMENTS

Melissa Hunter and Yijie Wang contributed equally to this article. Melissa Hunter is a Parker B. Francis Fellow in Pulmonary Research. This work was supported in part by a grant from the National Heart, Lung, and Blood Institute of by (R01 HL067176).

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Abbreviations: ApoE: apolipoprotein E, CCL: C-C motif ligand, Erk: extracellular-regulated kinase, GM-CSF: granulocyte-macrophage colony-stimulating factor, IPF: idiopathic pulmonary fibrosis, IL: interleukin, LPS: lipopolysaccharide, M-CSF: macrophage colony-stimulating factor, MAPK: mitogen-activated protein kinase, PTEN: phosphatase and tensin homolog, PI3K: phosphatidylinositide 3´ kinase, PDK: phosphoinositide-dependent protein kinase, ROS: reactive oxygen species, RNS: reactive nitrogen species, SHIP: SH2-containing inositol phosphatase, SHP2: SH2-containing phosphatase-2, STAT: signal transducer and activator of transcription, TGFβ: transforming growth factor β

Key Words: M-CSF, Monocyte, Macrophage, Pulmonary Fibrosis, Atherosclerosis, Breast Cancer, Lung Cancer, PI3K, AKT, Review

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