

Monocytes in Sterile Inflammation: Recruitment and Functional Consequences

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Abstract Monocytes play an important role in initiating innate immune responses. Three subsets of these cells have been defined in mice including classical, nonclassical and intermediate monocytes. Each of these cell types has been extensively studied for their role in infectious diseases. However, their role in sterile injury as occurs during ischemia–reperfusion injury, atherosclerosis, and trauma has only recently been the focus of investigations. Here, we review mechanisms of monocyte recruitment to sites of sterile injury, their modes of action, and their effect on disease outcome in murine models with some references to human studies. Therapeutic strategies to target these cells must be developed with caution since each monocyte subset is capable of mediating either anti- or pro-inflammatory effects depending on the setting.

Keywords Monocytes · Sterile inflammation · Recruitment · Functional outcome

Introduction

Monocytes are myeloid-derived cells originating in the bone marrow and maintaining residence in both the peripheral blood and the spleen. These cells, also called mononuclear blood phagocytes, are critical in the

inflammatory response to various stimuli, giving rise to tissue-resident macrophages and dendritic cells (DCs). In the mouse, two subtypes of monocytes exist for which there are seemingly corresponding human populations. Classical monocytes in the mouse are characterized by high expression of Ly6C and the chemokine receptor CCR2 as well as intermediate expression of CD43 (CD14⁺⁺CD16⁻ in humans), while a nonclassical monocyte population has low expression of both Ly6C and CCR2, but high expression of CD43 and a separate chemokine receptor, CX₃CR1 (CD14⁺CD16⁺⁺ in humans). In addition, intermediate monocytes have the phenotype Ly6C high and CD43 high (CD14⁺⁺CD16⁺ in humans) (Geissmann et al. 2003; Ziegler-Heitbrock et al. 2010).

Nonclassical Monocytes

Geissmann et al. (2003) described these cells as resident monocytes or Ly6C⁻CCR2⁻CD11b^{hi}CX₃CR1^{hi} cells situated along the endothelium with the role of “patrolling” the vasculature. The same paper termed the other subset inflammatory monocytes. In an effort to avoid the use of functional terms in describing these cells and to create uniformity in the field, a group of monocyte biologists convened and came to the consensus of renaming “inflammatory” cells classical monocytes and “resident” cells nonclassical monocytes (Ziegler-Heitbrock et al. 2010). It has been determined that nonclassical monocytes arise from classical monocytes upon downregulation of Ly6C and are thus the more mature of the two subsets (Sunderkotter et al. 2004). The ligand for CX₃CR1, CX₃CL1 or fractalkine, is unique among chemokines in that it is bound to a mucin-like stalk, allowing this chemotactic molecule to be bound to the surface of endothelial

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cells while at the same time extending far enough into the vasculature to make contact with circulating cells (Fong et al. 2000). Unlike classical monocytes, nonclassical monocytes are recruited to tissues even in the absence of inflammation (Geissmann et al. 2003). Nonclassical monocytes have been reported to rapidly extravasate into tissues. One report suggested that these cells are among the first responders to tissue injury and are able to produce both tumor necrosis factor (TNF)- α and interleukin (IL)-1 β (Auffray et al. 2007). Nonclassical monocytes are capable of being converted into specialized phagocytic cell types including CD11c⁺ MHC-II⁺ dendritic cells in the spleen as well as macrophages in the lung (Geissmann et al. 2003; Landsman and Jung 2007).

Classical Monocytes

Classical monocytes are characterized by high expression of Ly6C, CCR2, and CD11b with intermediate expression of CX₃CR1. These monocytes are recruited into inflamed tissue where they are capable of producing both TNF- α and IL-1 β . Monocyte emigration from the bone marrow is largely dependent on the chemokine CCL2 (MCP-1) and its receptor CCR2 (Nahrendorf et al. 2007; Serbina and Pamer 2006). Following transendothelial migration, classical monocytes differentiate into either DCs with increased expression of both CD11c and MHC class II or macrophages (Arnold et al. 2007; Geissmann et al. 2003). In vivo experiments have shown that monocyte-derived DCs are capable of inducing CD8⁺ T cell proliferation and regulate activation of CD4⁺ T cells (Geissmann et al. 2003; Gelman et al. 2010). Following surgical injury, monocytes are able to produce the neutrophil chemokine, CXCL2, but do not contribute to expression of CXCL1 (Armstrong et al. 2004).

Response to Infectious Agents

The majority of the literature regarding the role of monocytes responding to infectious pathogens deals with the importance of classical monocytes. However, there is evidence to suggest that CX₃CR1^{hi} nonclassical monocytes are important in defense against *Salmonella typhimurium*, a pathogenic intestinal bacterium. The role of nonclassical monocytes in response to this pathogen is thought to be in their conversion to tissue-resident DCs expressing high levels of CX₃CR1. These DCs are able to protrude through the intestinal wall, engulf bacteria, and transport the organism to lymph nodes (Niess et al. 2005). Activation of resident macrophages and DCs in the setting of other mucosal infections such as respiratory syncytial virus and

Legionella pneumophila is also enhanced in the presence of CX₃CL1 signaling (Johnson et al. 2012; Kikuchi et al. 2005).

Classical monocytes have clearly been shown to be important in response to infection. The elimination of several types of pathogens including bacteria (*L. monocytogenes*, *M. tuberculosis*), protozoa (*T. gondii*), and fungi (*A. fumigatus*) is impaired in mice deficient in CCR2. In response to these infections, classical monocytes can be converted into Tip-DCs (TNF- α and inducible nitric oxide synthase-producing DCs) or directly secrete inflammatory mediators including nitric oxide and IL-12. The role of monocytes in the defense against microbial pathogens has been reviewed extensively (Ingersoll et al. 2011; Serbina et al. 2008).

Sterile Inflammation

Inflammation is generally thought to occur in response to a pathogenic stimulus. However, many cases of “sterile” inflammation (in the absence of microbial or other infectious material) are known to occur including trauma, atherosclerosis and ischemia–reperfusion injury (as in cases of stroke, myocardial infarction and transplantation) (Chen and Nunez 2010).

Immune responses to infectious pathogens are initiated and amplified by the detection of pathogen-associated molecular patterns, molecules commonly found on the surface of bacteria or released by viruses. Innate immune cells express pattern-recognition receptors, including Toll-like receptors (TLRs), NOD-like receptors, and RIG-I like receptors that bind these molecules and activate innate immune cells to defend against the intruding pathogen (Medzhitov 2001). These same receptors have also been shown to be important in initiating immune responses to injury occurring in a sterile environment, in which case the stimuli are termed damage-associated molecular patterns (DAMPs). Molecules normally sequestered within healthy cells but released upon cell death act as stimuli and include DNA, RNA, high-mobility group box 1, heat shock proteins (HSP), and cholesterol crystals (Chen and Nunez 2010; Tsan and Gao 2004). As in infectious inflammation, activation of immune cells by DAMPs leads to increased expression of inflammatory cytokines and the recruitment of neutrophils and monocytes.

While the role of monocytes in infectious inflammation has been the topic of many reviews (Serbina et al. 2008; Shi and Pamer 2011) the role of monocytes in sterile inflammation is only recently being appreciated. Although analogies exist between the recruitment of monocytes in sterile and infectious inflammation, there are additional functions of monocytes in repair and healing that are more

documented in noninfectious inflammation. Here, we will review the role of monocytes in various examples of sterile inflammation in murine systems including the method of their recruitment, mechanisms of action and consequences of their activation.

Mechanisms of Monocyte Recruitment During Sterile Inflammation

CCL2 (monocyte chemoattractant protein-1) has been shown to be important in recruiting classical monocytes out of the bone marrow and into the bloodstream (Fig. 1). In a paper investigating monocytes in the ischemic liver, monocyte recruitment into the organs of mice lacking CCR2 was virtually eliminated correlating with a concomitant increase of monocytes in the bone marrow (Bamboot et al. 2010). Similar findings have been seen in models of atherosclerosis, in which CCR2 knockout (KO) mice have increased numbers of monocytes in the bone marrow compartment, but decreased numbers at the site of inflammation (Tsou et al. 2007). This is an indication that monocytes recruited to sites of sterile inflammation originate in the bone marrow. A recent paper investigating the

effect of the chemotherapeutic drug, cyclophosphamide, demonstrated that classical monocyte egress from the bone marrow is affected in an opposite manner by the CX₃CL1–CX₃CR1 axis (Jacquelin et al. 2013). While CCL2 drives inflammatory monocyte emigration from this compartment, CX₃CL1 causes these cells to be retained in the bone marrow. In this study, nonclassical monocyte egress from the bone marrow was not affected by the absence of either of these chemokine receptors.

Some studies suggest that recruitment of classical monocytes from the bloodstream to sites of sterile inflammation is largely dependent on the expression of CCL2. This chemokine has been shown to not only cause monocyte chemotaxis but also firm adhesion onto endothelial monolayers (Gerszten et al. 1999; Maus et al. 2002). In a rat model of traumatic brain injury, monocytes migrated across the blood–cerebrospinal fluid barrier in response to CCL2 produced by the choroid plexus epithelial cells (Szmydynger-Chodobska et al. 2012). In another sterile injury model using cerebral ischemia, CCR2-deficient mice have decreased recruitment of classical monocytes (Dimitrijevic et al. 2007). In vitro studies showed that CCL2 was expressed by both brain microvascular endothelial cells and astrocytes (Dimitrijevic et al.

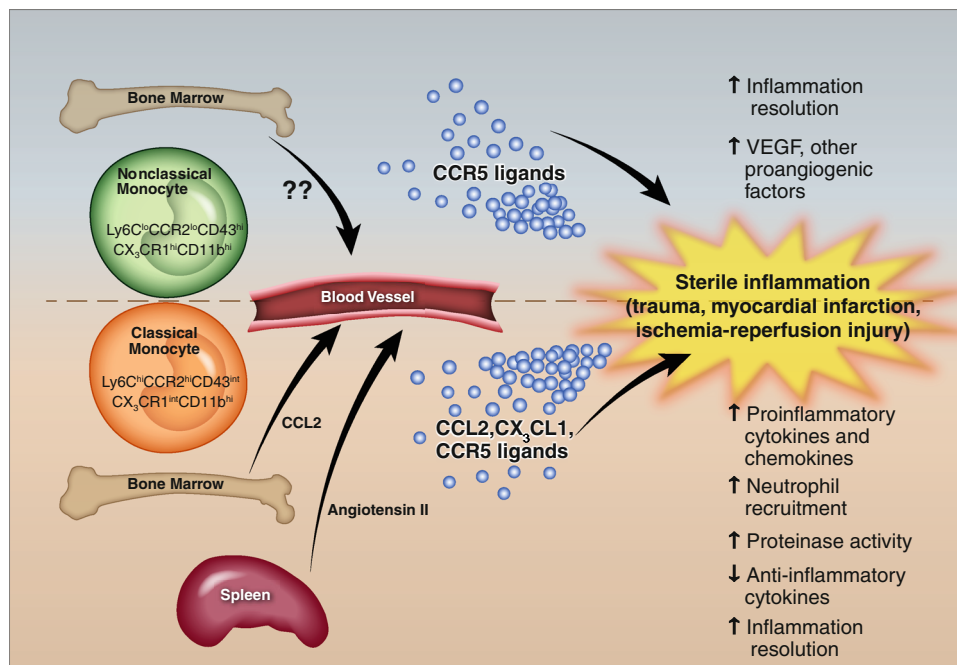


Fig. 1 Two distinct subtypes of murine monocytes exist, nonclassical (Ly6C^{lo}CCR2^{lo}CD43^{hi}CX₃CR1^{hi}CD11b^{hi}) and classical (Ly6C^{hi}CCR2^{hi}CD43^{int}CX₃CR1^{int}CD11b^{hi}). The nonclassical subset originates in the bone marrow and is recruited to sites of inflammation by CCR5 ligands. Classical monocytes reside in the bone marrow, but also have a reservoir in the spleen. Egress from the bone marrow is dependent on CCR2 ligands, while migration out of the spleen is dependent on Angiotensin II. Upon entering the bloodstream, they are

recruited to sites of inflammation through CCL2, CX₃CL1, and CCR5 ligands. While nonclassical monocytes play an important role in the resolution of inflammation and secreting vascular endothelial growth factor (VEGF) and other pro-angiogenic factors, classical monocytes increase production of pro-inflammatory cytokines and chemokines, cause neutrophil recruitment, lead to an increase in proteinase activity, and can also resolve inflammation

2006). Surprisingly, endothelial cells in this setting also expressed the receptor CCR2, and ligation with CCL2 caused tight junctions in endothelial cells to be disrupted increasing vascular permeability. The CCR2–CCL2 axis in this system has developed to not only directly recruit monocytes to the injured tissue, but also to further facilitate entry of these cells by causing disruption of the endothelial barrier. However, lung transplant studies have shown that while the emigration of monocytes from the bone marrow to the peripheral blood is dependent on CCR2, their migration into graft tissue is independent of this chemokine, which mirrors observations in a model of infection with *Listeria monocytogenes* (Gelman et al. 2010; Serbina and Pamer 2006).

In addition to the bone marrow, the spleen also provides an important monocyte reservoir. By two separate techniques, Swirski et al. (2009) showed that splenic monocytes rather than bone marrow-derived monocytes populated the ischemic myocardium. Consistent with the notion that CCR2 is necessary for monocyte release from the bone marrow but not from the spleen, monocytes were efficiently released into the bloodstream in CCR2KO mice. In addition, splenectomizing mice prior to ischemia led to an inability of monocytes to populate the inflamed heart tissue (Swirski et al. 2009). Moreover, this study showed the importance of angiotensin II in splenic monocyte egress. A separate study also using myocardial infarction showed that monocytopoiesis in the spleen was dependent on the inflammatory cytokine IL-1 β (Leuschner et al. 2012).

Several studies have examined the role of CX₃CR1 in the recruitment of monocytes during sterile inflammation. Tacke et al. (2007) showed some surprising mechanisms of both classical and nonclassical monocyte recruitment in a mouse model of atherosclerosis. ApoE^{-/-} mice fed a high fat diet are susceptible to atherosclerotic lesions and have an increased abundance of monocytes in the blood. This group designed methods for labeling either classical or nonclassical monocytes using fluorescent latex beads. Specific labeling of nonclassical monocytes was achieved by observing bead positive monocytes 8 h after injection of fluorescent beads. While both subsets of monocytes initially took up these beads, at 8 h, labeling was restricted to only the nonclassical monocytes. Alternatively, specific labeling of classical monocytes was achieved by first depleting all monocytes with clodronate liposomes followed 24 h later by injection of fluorescent beads. Upon monocyte return, latex bead labeling was restricted to Ly6C^{hi} classical monocytes. Using these methods of monocyte labeling, Tacke et al. (2007) found that both CCR2 and CX₃CR1 were required for migration of classical monocytes into atherosclerotic lesions. However, neither of these two chemokine receptors was required for

nonclassical monocyte migration, which was dependent on CCR5 (Tacke et al. 2007).

In a model of renal ischemia reperfusion injury, Oh et al. (2008) showed that the chemokine CX₃CL1 was expressed by kidney endothelial cells within 8 h of reperfusion. This group also provided in vitro evidence that the increased expression of CX₃CL1 was due to exposure of endothelial cells to hypoxic conditions. Blocking the interaction of this chemokine with its receptor using a CX₃CR1 antibody decreased the number of CD11b⁺ cells (which the authors determined to be macrophages) infiltrating the kidney. In a separate model using cardiac allograft rejection, expression of CX₃CL1 continued to increase on graft endothelium up to 7 days post-transplant (Robinson et al. 2000). In vitro studies performed by this group demonstrated that the increased chemokine expression on endothelial cells was due to exposure to TNF- α . Furthermore, CX₃CL1 mediated adhesion of peripheral blood mononuclear cells to endothelial cells and treatment of murine heart transplant recipients with neutralizing anti-CX₃CR1 antibodies resulted in prolonged allograft survival.

Other methods of monocyte recruitment to sites of sterile injury have been investigated including a role for matrix metalloproteinase-9 (MMP-9) and CXCL12. Zhang and colleagues (2011) showed that CXCL12 contributes to the recruitment of monocytes to sites of spinal cord injury. Upon entering the tissue, monocytes produced MMP-9, potentially causing the breakdown of the endothelial basement membrane and therefore allowing transmigration of the cells. In these experiments, drugs targeting MMP-9 and CXCL12 as well as the absence of these molecules in genetically deficient mice resulted in decreased recruitment of monocytes to the site of injury (Zhang et al. 2011). Local production of TNF- α at the site of injury may have triggered the expression of MMP-9. Thus, a complex system of chemokines, cytokines and proteolytic enzymes was proposed to be responsible for monocyte migration into the inflamed tissue.

Mechanisms of Monocyte Action During Sterile Inflammation

As monocytes quickly differentiate into macrophages or DCs upon leaving the bloodstream, it is difficult to isolate monocyte function from that of their tissue-resident counterparts. That being said, the literature does contain various references of direct functions of monocytes in sterile inflammation.

In models of sterile injury in mice deficient in CCR2, where recruitment of monocytes is impaired, levels of pro-inflammatory mediators at the site of damage are significantly altered. Compared to wild-type mice, CCR2 KO

mice undergoing cerebral ischemia reperfusion injury had decreased expression of the neutrophil chemoattractant CXCL5 as well as the pro-inflammatory cytokines IL-1 β and TNF- α at the site of inflammation. In contrast, expression of cytokines such as IL-4, IL-5 and IL-13 was increased in CCR2 KO animals (Dimitrijevic et al. 2007). While experiments performed in this study do not definitively point to monocytes as the producers of these inflammatory modulators, it does demonstrate that monocytes have a profound effect on the local inflammatory state.

Evidence of direct monocyte expression of pro-inflammatory cytokines has been shown in ex vivo-stimulated monocytes derived from humans following myocardial infarction. These patients have increased serum levels of HSP70, which as mentioned above can act as a DAMP. This endogenous activator of the innate immune system is released upon cardiac damage and can bind to TLR4. Monocytes isolated from patients post-myocardial infarction and incubated with different concentrations of HSP70 show increased expression of inflammatory cytokines including IL-6 and TNF- α . Blocking TLR4 in these experiments inhibited monocyte production of these cytokines (Satoh et al. 2006). Mouse models have also demonstrated the importance of monocytes in directly contributing to the inflammatory milieu. Classical monocytes infiltrating livers following ischemia reperfusion injury have increased expression of reactive oxygen species, TNF- α and IL-6 (Bamboate et al. 2010). Depletion of CD11c⁺ DCs resulted in increased expression of these pro-inflammatory mediators specifically in Ly6C^{hi} monocytes suggesting that DCs control detrimental effects of classical monocytes during liver ischemia. In this model, monocytes were largely responsible for ischemia reperfusion injury-mediated liver damage (Bamboate et al. 2010).

Regulation of such inflammatory cytokines may in part be through the expression of two interacting proteins, the arrestin protein, ARRB2, and I κ B in monocytes. In a renal transplant model, monocytes accumulate in large numbers in the vasculature of rejecting allografts compared to isografts (Grau et al. 2001). In these monocytes, decreased expression of ARRB2 and I κ B as measured by immunoblotting was associated with increased NF κ B signaling and downstream expression of IL-1 β and TNF- α (Zakrzewicz et al. 2011).

Monocytes may further contribute to inflammation through the recruitment of neutrophils, which are known to be important mediators of tissue injury during inflammation. Early in vitro studies showed that hypoxic conditions associated with ischemia reperfusion injury caused monocytes to express neutrophil chemoattractants (Metinko et al. 1992). Recent intravital two-photon microscopy studies performed in our laboratory uncovered a role for

monocytes in the recruitment of neutrophils into reperfused lung grafts (Kreisel et al. 2010). Injection of clodronate liposomes, which deplete blood-borne phagocytic cells including monocytes, resulted in the inability of neutrophils to traffic into the ischemic lung tissue despite being capable of adhering to the endothelium. This indicates that monocytes regulate the transendothelial migration of neutrophils into sites of sterile inflammation. Furthermore, neutrophils frequently co-localized with monocytes in the transplanted lung tissue raising the possibility that monocytes provide chemotactic cues to neutrophils following their extravasation.

However, monocyte function is not always pro-inflammatory. Activated intravascular monocytes within transplanted renal grafts during rejection may also play a role in dampening inflammation through expression of nonneuronal acetylcholine, which can attenuate ATP-mediated signaling in autocrine or paracrine fashion (Hecker et al. 2009). During spinal cord injury, monocytes can have a protective role regulating the resolution of scar tissue. These cells are converted into “resolving/regulatory” macrophages expressing both IL-10 and MMP-13 (Shechter et al. 2009, 2011). Expression of IL-10 by monocytes led to an overall anti-inflammatory state that limited lesion size and prevented resident microglial cell activation (Shechter et al. 2009). Monocytes in spinal cord injury also regulate scar resolution by secreting MMP-13 in response to chondroitin sulfate proteoglycan, a component of the glial scar matrix (Shechter et al. 2011).

Nahrendorf et al. (2007) investigated the role of both classical and nonclassical monocytes in response to myocardial infarction. After cardiac injury, these two subsets were recruited to the tissue in two waves: an initial phase, during which classical monocytes were primarily recruited and a secondary phase during which nonclassical monocytes predominated. During the first phase, Ly6C^{hi}CCR2^{hi} monocytes exhibited high proteinase activity (presumably to break down the extracellular matrix) as well as pro-inflammatory TNF- α . Ly6C^{lo}CX₃CR1^{hi} monocytes, however, were more important in healing, expressing vascular endothelial growth factor and other pro-angiogenic factors (Nahrendorf et al. 2007).

Monocyte Effect on Outcome of Disease During Sterile Inflammation

For the majority of examples of sterile inflammation, monocytes have been shown to be detrimental to the outcome of disease. In cases of cerebral ischemia (Dimitrijevic et al. 2007), bronchiolitis obliterans syndrome associated with tracheal transplantation (Belperio et al. 2001) and atherosclerosis (Boring et al. 1998),

absence or disruption of the CCR2–CCL2 axis was associated with improved outcomes. A recent patient study investigating pancreatic cancer demonstrated that higher monocyte abundance in the blood was correlated with decreased survival over a 5-year period. These monocytes infiltrated the tumors where they presumably became tumor-associated macrophages (TAM) capable of suppressing infiltrating CD8⁺ T cells. In the same study, a mouse model of pancreatic cancer showed that treatment with a CCR2 inhibitor promoted antitumor immunity associated with a decrease in TAMs and concomitant increase in tumor-infiltrating effector T cells (Sanford et al. 2013).

However, evidence also exists for a role of classical monocytes in the improvement of outcome. Several studies examining skeletal muscle injury illustrated an important role for CCR2^{hi} monocytes in the repair of tissue. Models using freeze-induced injury and injection of cardiotoxin have demonstrated that recruitment of monocytes (presumably the classical subset due to the use of CCR2 KO mice) improves the functional strength of recovering muscles (Warren et al. 2004, 2005). In the case of cardiotoxin-induced injury, CCR2 KO mice had delayed cellular infiltrates, impaired muscle regeneration, and increased intermuscular adipocytes (Martinez et al. 2010). Furthermore, monocytes (as well as other bone marrow cells and muscle tissue) expressed CCL2 leading to increased expression of insulin-like growth factor, which was important in the healing process (Lu et al. 2011). As discussed above, separate studies on spinal cord injury have shown that infiltration of monocytes to the site of damage is necessary for both controlling inflammation and remodeling of scar tissue (Shechter et al. 2009, 2011).

The literature regarding the effects of nonclassical monocytes on outcomes of diseases is more ambiguous. Studies examining cerebral (Soriano et al. 2002) and renal (Furuichi et al. 2006) ischemia reperfusion injury showed that the absence of CX₃CR1 signaling correlated with decreased inflammation. In the study of renal ischemia reperfusion injury, absence of this signaling pathway was also associated with less fibrosis. However, other studies indicate that the CX₃CL1–CX₃CR1 axis is associated with improved outcomes. Louvet et al. (2004) demonstrated that increased expression of CX₃CL1 and its receptor are linked with cardiac allograft tolerance. As mentioned above, CX₃CR1^{hi} monocytes were important in the healing myocardium after infarction (Nahrendorf et al. 2007). Finally, one other study, while not implicating monocytes in the effect, showed that CX₃CL1 expression was important in angiogenesis and recovery after hindlimb ischemia (Ryu et al. 2008). These reports illustrate the importance of using prudence when considering blockade of these pathways for clinical interventions.

Conclusion

Presently, there are few drugs to antagonize either CCR2 or CX₃CR1. Two drugs, RO5234444 and RAP-103, also block the CCR5 receptor and are in pre-clinical trials. Two studies have shown efficacy for these orally active small molecule antagonists. RO5234444 showed improved outcomes in diabetic nephropathy (Sayyed et al. 2011) while RAP-103 attenuated rodent neuropathic pain (Padi et al. 2012). A separate drug called PF-04136309 is a CCR2 kinase antagonist from Pfizer (Xue et al. 2010). Sanford et al. (2013) are beginning Phase Ib/II clinical trials with this drug in pancreatic cancer to block classical monocyte infiltration into tumors.

The role of monocytes in sterile inflammation is only starting to be fully appreciated. These cells are obviously very important in exacerbating inflammation in several models of sterile inflammation. However, they may also be critical in the later phases of healing. In addition, the role of monocytes may differ based on different anatomic locations and the nature of the inflammatory stimuli. With this in mind, it will be important to consider the possibility of monocyte inhibition causing both favorable and unfavorable results in different clinical settings.

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