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Monocyte-Mediated Immune Defense Against Murine *Listeria monocytogenes* Infection

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Abstract

Infection of mice with *Listeria monocytogenes* induces a robust innate inflammatory response that restricts bacterial growth in the liver and spleen prior to the development of protective T cell responses. Ly6C^{hi} monocytes contribute to the innate immune response following *L. monocytogenes* infection and in their absence, mice rapidly succumb to infection. Emigration of Ly6C^{hi} monocytes from the bone marrow into the circulation is the first step in their recruitment to sites of *L. monocytogenes* infection and is triggered by CCL2- and CCL7-mediated stimulation of CCR2 chemokine receptors on monocytes. CCL2 expression by mesenchymal stem cells in the bone marrow, in response to TLR stimulation, drives monocyte emigration from cellular compartments into vascular sinuses of the bone marrow. In addition to TLR ligands, type I interferon-mediated signals can also drive monocyte emigration from the bone marrow during *L. monocytogenes* infection. Once Ly6C^{hi} monocytes enter the bloodstream, trafficking to sites of infection in the liver and spleen is CCR2 independent. In the liver, CD11b on the monocyte and ICAM-1 on the surface of endothelial cells target Ly6C^{hi} monocytes to foci of *L. monocytogenes* infection. At the site of infection, Ly6C^{hi} monocytes undergo MyD88-dependent differentiation into TNF and iNOS-producing dendritic cells (TipDCs) and express MHC class II, B7.1, and CD40 on their cell surface. How TipDCs mediate bacterial clearance during early *L. monocytogenes* infection remains an active area of investigation.

1. MONOCYTES AND THE DISCOVERY OF *LISTERIA MONOCYTOGENES*

A potentially important role for monocytes in defense against *L. monocytogenes* infection was suggested as early as 1926 when investigators from Cambridge University described a lethal infection of rabbits that was caused by a previously unknown Gram-positive bacterium. Because these rabbits developed a marked monocytosis, the organism was named *Bacterium monocytogenes* (Murray *et al.*, 1926). A report the following year described a lethal infection of gerbils in South Africa, again caused by an unknown Gram-positive bacterium. This organism was named *Listerella hepatolytica; Listerella* in honor of Sir Joseph Lister (who had died 15 years earlier) and *hepatolytica* because this bacterium caused hepatic necrosis (Pirie, 1927). Subsequent studies determined that *B. monocytogenes* and *L. hepatolytica* are the same organism and bacterial taxonomists eventually settled on

the name *L. monocytogenes.* Another 60 years passed before the role of monocytes in defense against *L. monocytogenes* infection was more completely characterized.

2. MONOCYTE POPULATIONS CIRCULATING IN THE MOUSE BLOODSTREAM

The past 5 years have seen great advances in our understanding of circulating monocytes, a subset of blood leukocytes that until recently received far less attention than other circulating white blood cells (Auffray *et al.,* 2009a,b). In mice, chemokine receptor expression has been used to distinguish distinct monocyte subsets that differ in terms of trafficking under homeostatic and inflammatory/infectious conditions (Geissmann *et al.,* 2003; Palframan *et al.,* 2001). One subset of circulating monocytes expresses the Ly6C surface marker and the CCR2 chemokine receptor. This subset traffics to sites of inflammation and infection, and these cells are therefore often referred to as inflammatory or Ly6C^{hi} monocytes (Auffray *et al.,* 2009a,b). A second subset of murine monocytes is distinguished by high expression of the CX3CR1 chemokine receptor and low Ly6C expression (Geissmann *et al.,* 2003). These cells, referred to as CX3CR1^{hi} or Ly6C^{lo} monocytes, have distinct trafficking characteristics, which include LFA-1-dependent patrolling on the luminal surface of small blood vessels (Auffray *et al.,* 2007). Their role in defense against infectious diseases is less well defined, although they have been implicated in very early responses to *L. monocytogenes* infection (Auffray *et al.,* 2009a,b).

3. CCR2-MEDIATED MONOCYTE RECRUITMENT

Inflammatory monocyte recruitment to sites of infection is mediated by CCR2 on the monocyte, and the chemokines CC-chemokine ligand 2 (CCL2, also referred to as MCP1) and CCL7 (also known as MCP3) (Tsou *et al.,* 2007). Many nucleated cells can express CCL2 in response to a range of stimuli (Brown *et al.,* 1994; Rollins and Pober, 1991; Struyf *et al.,* 1998; Tsou *et al.,* 2007; Tsuboi *et al.,* 2002). The dramatic induction of CCL2 expression during infection, in part triggered by stimulation of TLRs by microbial molecules (Tsuboi *et al.,* 2002), suggested that CCR2-expressing monocytes follow a gradient of CCL2 from the bloodstream to the infected focus. CCL2, like many chemokines, is known to dimerize and associate with glycosaminoglycans (GAGs), and some experimental evidence supports the notion that association with GAGs is required for CCL2's *in vivo* activity (Allen *et al.,* 2007; Proudfoot *et al.,* 2003). Although much less studied, CCL7 also contributes to Ly6C^{hi} monocyte recruitment (Jia *et al.,* 2008; Tsou *et al.,* 2007) and will be discussed in greater detail in later sections. While chemokines are believed to provide monocytes with a sense of direction during their recruitment, the mechanics of recruitment are mediated by integrins and other adhesion molecules (Ley *et al.,* 2007).

4. MYELOID CELLS IN DEFENSE AGAINST *L. MONOCYTOGENES*

The importance of myeloid cells in defense against *L. monocytogenes* infection was first demonstrated by Rosen and colleagues, in studies using the 5C6 monoclonal antibody, which blocks CD11b and thus prevents inflammatory cell trafficking into infected tissues. In this study, administration of this blocking antibody at the initiation of infection resulted in

uncontrolled growth of *L. monocytogenes* in the livers of infected mice, while blockade at later stages of infection was far less immunocompromising (Rosen *et al.*, 1989). Histologic examination of the livers of 5C6-treated mice following infection demonstrated markedly reduced myeloid cell infiltration and far greater hepatic necrosis and bacterial growth, leading the investigators to conclude that these myeloid cells restrict infection of hepatocytes. This study, however, could not distinguish between monocytes and neutrophils, as trafficking of both of these populations was inhibited by CD11b blockade. A subsequent study by Kurihara and colleagues investigated *L. monocytogenes* infection of mice genetically engineered to lack the CCR2 chemokine receptor (Kurihara *et al.*, 1997). Systemic infection of CCR2-deficient mice with *L. monocytogenes* demonstrated their marked susceptibility, with greatly reduced clearance of bacteria from the spleen and liver and far greater hepatic necrosis. These authors recognized that CCR2 deficiency resulted in reduced recruitment of mononuclear phagocytes to sites of infection and demonstrated the importance of this cell population and this chemokine receptor in defense against *L. monocytogenes.*

5. LY6C^{HI} MONOCYTES AND TIPDCS DURING *L. MONOCYTOGENES* INFECTION

Although the roles of CCR2 and CD11b in defense against *L. monocytogenes* were well established by these studies, the cells that mediate antibacterial defense during the early stages of infection remained incompletely defined. To begin a characterization of monocyte-mediated antimicrobial defense, wild-type and CCR2-deficient mice were infected with *L. monocytogenes* and inflammatory cell recruitment to the spleen over the first 3 days of infection was investigated (Serbina *et al.*, 2003b). These studies demonstrated that *L. monocytogenes* infection resulted in the recruitment of CD11b and Mac-3 expressing cells in wild-type mice and that the frequency of these cells was markedly reduced in CCR2-deficient mice. CD11b^{+}Mac-3^{+} cells also expressed low levels of CD11c and morphologically resembled dendritic cells. Further characterization of these cells demonstrated that they produced high levels of TNF and iNOS and expressed CD40, B7.1, B7.2, and high levels of MHC class II on the cell surface. Because these cells were able to prime naïve alloreactive T cells and produced TNF and iNOS, they were named TNF/iNOS-producing dendritic cells (TipDCs). A remarkable characteristic of these cells is that they were recruited to foci of *L. monocytogenes* infection in the white pulp of the spleen and, although not directly infected, were the major producers of TNF and iNOS. Thus, in CCR2-deficient mice, splenic TNF and iNOS levels were markedly reduced following *L. monocytogenes* infection. As both TNF and iNOS had been demonstrated in previous studies to be essential for clearance of murine *L. monocytogenes* infection (MacMicking *et al.*, 1995; Pfeffer *et al.*, 1993; Rothe *et al.*, 1993), the identification of TipDCs as their source provided new insight into cellular mechanisms of antimicrobial defense. Further, the finding that TNF and iNOS are predominantly produced by cells that do not harbor live bacteria suggested that stimuli from infected cells and the release of microbial products from foci of infection drive the production of these critical immune mediators. The ability of CCR2-expressing monocytes to confer resistance to *L. monocytogenes* infection was demonstrated by their adoptive transfer into infected, CCR2-deficient recipient mice. In these experiments,

adoptive transfer of CCR2-expressing monocytes enhanced bacterial clearance from the spleen (Serbina *et al.*, 2003b).

6. THE ROLE OF CCL2 AND CCL7 IN MONOCYTE RECRUITMENT

Recruitment of TipDCs to the spleen was CCR2 dependent, suggesting that *L. monocytogenes* infection induced the expression of CCR2 ligands, such as CCL2. Indeed, earlier studies had demonstrated that *L. monocytogenes* infection is accompanied by *in vivo* expression of chemokines, including CCL2 (Barsig *et al.*, 1998). *In vivo* production of CCL2, however, required inoculation of mice with strains of *L. monocytogenes* that gain access to the host cell cytosol (Serbina *et al.*, 2003a). Thus, infection of mice with strains of *L. monocytogenes* that lacked listeriolysin-O, the essential virulence factor that enables bacteria to escape the vacuole and enter the cytosol, did not induce detectable levels of CCL2 expression in the spleen. Similarly, inoculation of mice with heat-killed *L. monocytogenes* also did not induce CCL2 expression. However, infection of mice with an attenuated strain of *L. monocytogenes* lacking ActA, a virulence factor that facilitates actin polymerization and intracytoplasmic motility, induced *in vivo* CCL2 expression. ActA-deficient *L. monocytogenes*, though attenuated, accesses the cytoplasm of host cells, suggesting that this step is essential for induction of CCL2 during *in vivo* infection. Consistent with this result, TipDC recruitment to the spleen is normal in mice inoculated with ActA-deficient *L. monocytogenes*, while neither LLO-deficient nor heat-killed *L. monocytogenes* administration resulted in TipDC recruitment to the spleen.

To determine whether CCL2 contributes to defense against *L. monocytogenes* infection, CCL2-deficient mice were infected and the course of infection was monitored. These studies demonstrated that CCL2 contributes to bacterial clearance, although CCL2-deficient mice are not as sensitive to *L. monocytogenes* infection as CCR2-deficient mice. TipDC recruitment to the spleen of CCL2-deficient mice is reduced by approximately 60%, suggesting that the intermediate phenotype of CCL2-deficient as opposed to CCR2-deficient mice results from reduced but not absent recruitment of this cell population to sites of infection (Serbina *et al.*, 2003a). This result also suggested that other chemokines can stimulate CCR2-mediated Ly6C^{hi} monocyte recruitment during *L. monocytogenes* infection.

To address this possibility, CCL7-deficient mice were investigated. CCR2 also responds to stimulation by CCL7, and mice deficient for CCL7 have diminished recruitment of Ly6C^{hi} monocytes to sites of inflammation (Tsou *et al.*, 2007). Murine infection with *L. monocytogenes* induces CCL7 expression in serum, blood, liver, and kidney with kinetics that are similar to CCL2 expression (Jia *et al*, 2008). Tissue levels of CCL7 and CCL2 differ, however, with the amount of CCL7 exceeding the amount of CCL2 in the kidneys of infected mice. Peak chemokine concentrations are present in these tissues approximately 48 h after intravenous infection, correlating with the peak of bacterial infection. Similar to CCL2 induction by *L. monocytogenes* infection, only cytoplasm invasive strains of *L. monocytogenes* induce *in vivo* expression of CCL7. Thus, inoculation of mice with LLO-deficient and heat-killed bacteria did not induce CCL7 expression. Along similar lines, tissue culture bone marrow macrophages produce CCL7 in response to virulent *L. monocytogenes* infection, but induction of CCL7 is markedly reduced in response to LLO-

deficient or heat-killed bacteria (Jia *et al.*, 2008). CCL7-deficient mice are more susceptible to *L. monocytogenes* infection. Similar to CCL2-deficient mice, mice lacking CCL7 are not as susceptible as CCR2-deficient mice, but they are more susceptible than wild-type mice. Infection of mice lacking CCL7 with virulent *L. monocytogenes* results in approximately 50% reduced recruitment of TipDCs to infected spleens when compared to infected wild-type mice. These results suggest that CCL2 and CCL7 make nonredundant contributions to CCR2-dependent recruitment of monocytes to sites of *L. monocytogenes* infection. It remains unclear, however, whether CCL2 and CCL7 contributions in wild-type mice are simply additive or whether these two chemokines function in physically distinct sites during infection.

7. CCL2 EXPRESSION DURING *L. MONOCYTOGENES* INFECTION

To begin to determine the upstream signaling pathways that trigger CCL2 expression in response to *L. monocytogenes* infection, MyD88-, TNF-, and Caspase-1-deficient mice were infected and chemokine levels were measured in the spleen. In comparison to wild-type mice, CCL2 expression in the infected spleens was modestly decreased in TNF-, Caspase-1 (and therefore IL-1)-, and MyD88-deficient mice (Serbina *et al.*, 2003a). MyD88 deficiency had been shown previously to markedly increase susceptibility to *L. monocytogenes* infection (Edelson and Unanue, 2002; Seki *et al.*, 2002). Recruitment of inflammatory monocytes to the spleen of MyD88-deficient mice following *L. monocytogenes* inoculation was not reduced during the first 2 days of infection. However, production of TNF and iNOS by recruited monocytes in the spleen of infected mice was markedly reduced. This study, therefore, demonstrated that monocyte recruitment, during the first 2–3 days following infection, is driven by a MyD88-independent, innate immune signaling pathway that is triggered by bacterial invasion of the host cell's cytoplasm. Differentiation of recruited monocytes into TipDCs, however, is MyD88 dependent and likely occurs as a result of TLR-mediated signals in response to microbial molecules at the site of infection (Serbina *et al.*, 2003a). *L. monocytogenes* infection induces splenic dendritic cells to recruit and cluster NK cells and inflammatory monocytes at sites of infection, and interferon-γ produced by NK cells contributes to MyD88-dependent differentiation of $Ly6C^{hi}$ monocytes into TipDCs (Kang *et al.*, 2008).

8. *L. MONOCYTOGENES* INFECTION INCREASES MONOCYTOPOIESIS

Recruitment of neutrophils and monocytes to peripheral sites is essential for defense against a number of microbial pathogens. Under homeostatic conditions, the frequency of these cells in peripheral tissues is low. Following infection, their numbers increase due, in part, to accelerated emigration from bone marrow and, in part, to enhanced recruitment into inflamed tissues. Although recruitment and activation of myeloid cells in peripheral tissues have been studied, relatively little is known about inflammation-driven bone marrow hematopoiesis. In order to address this issue, bone marrow myeloid populations were characterized following infection with *L. monocytogenes* (Serbina *et al.*, 2009). These studies demonstrated that inflammation induced by *L. monocytogenes* infection dramatically changes the hematopoietic compartment of the bone marrow and specifically promotes monopoiesis. In contrast to the effect of infection on monocyte production, granulocyte

production in the bone marrow was decreased during later stages of infection and, accordingly, monocytes and not granulocytes were increasingly recruited to infected spleen as infection progressed. In the absence of Myd88/Trif-mediated signaling, monocyte frequencies and proliferation of monocyte precursors were diminished in the bone marrow beyond 72 h of *L. monocytogenes* infection. This study demonstrated that proliferation and replenishment of monocyte precursors are dependant on ongoing inflammation and require TLR-mediated signaling. Thus, innate immune deficiency resulting from loss of Myd88 expression stems not only from inadequate cell activation during infection but also from the inability to replenish innate immune effector cells, in particular, $Ly6C^{hi}$ monocytes.

9. TYPE I INTERFERON EFFECTS ON CCL2 EXPRESSION

Infection of host cells by cytosol invasive *L. monocytogenes* results in the expression of type I interferon (O'Riordan *et al.*, 2002). Interferon-β and interferons-α signal via the type I interferon receptor (IFNAR), and type I interferon signaling is abolished in IFNAR-deficient mice. While type I interferon signaling is deleterious during *L. monocytogenes* infection (Auerbuch *et al.*, 2004; Carrero *et al.*, 2004; O'Connell *et al.*, 2004), this signaling pathway is capable of stimulating CCL2 expression (Hokeness-Antonelli *et al.*, 2007). To determine the relative contributions of MyD88 and IFNAR to the induction of CCL2 expression during *L. monocytogenes* infection, MyD88, IFNAR, and MyD88/IFNAR double-deficient mice were infected and CCL2 levels were measured in the spleen and serum (Jia *et al.*, 2009). These studies demonstrated that combined deletion of MyD88 and IFNAR signaling markedly reduced CCL2 production.

Analysis of bone marrow macrophages demonstrated that MyD88-mediated signals induced CCL2 mRNA within 30 min of infection and that IFNAR-mediated signals amplified CCL2 mRNA levels between 1 and 6 h after infection. $Ly6C^{hi}$ monocyte recruitment to spleens following *L. monocytogenes* infection was normal in IFNAR- and MyD88-deficient mice but was markedly reduced in mice with combined deficiency of IFNAR and MyD88. Thus, with respect to monocyte recruitment during *L. monocytogenes* infection, IFNAR and MyD88 signaling pathways provide some redundancy that ensures monocyte recruitment.

While MyD88-deficient mice are highly susceptible to *L. monocytogenes* infection, and IFNAR-deficient mice are more resistant, double-deficient mice are even more susceptible to infection than MyD88-deficient mice. This result suggests that type I interferon-mediated signals can be protective, but only in the absence of TLR signaling. Previous studies had demonstrated that *L. monocytogenes* infection induces T cell apoptosis in the white pulp of the spleen, inducing IL-10 production and downregulating innate immune defenses (Carrero *et al.*, 2004; Tripp *et al.*, 1993). Type I interferon induction during infection enhances T cell apoptosis and thus enhances IL10 production (Carrero *et al.*, 2004, 2006), thereby reducing innate immune resistance to *L. monocytogenes* infection. In IFNAR-deficient mice, T cell apoptosis following *L. monocytogenes* infection is reduced, thereby paradoxically enhancing resistance because IL-10 expression is presumably decreased. If IL-10 induction during *L. monocytogenes* infection is MyD88 dependent, then the immunocompromising effect of type I interferon expression would be negated by MyD88 deficiency. Thus, by eliminating

the immunocompromising effect, MyD88 deficiency reveals the protective effect of type I interferon signaling.

10. CCR2-DEFICIENT LY6C^{HI} MONOCYTES ARE RETAINED IN THE BONE MARROW

Recruitment of Ly6C^{hi} monocytes during *L. monocytogenes* infection is a multistep process that culminates in the arrival of TipDCs at sites of infection. Although it seemed likely that CCR2 would mediate the recruitment of circulating Ly6C^{hi} monocytes from the bloodstream to infected tissues, careful analysis of bloodstream monocyte frequencies in *L. monocytogenes*-infected, CCR2-deficient mice revealed a marked paucity of monocytes in the blood (Serbina and Pamer, 2006). Even more surprising, frequencies of Ly6C^{hi} monocytes in the blood of uninfected CCR2-deficient mice were also markedly reduced compared to uninfected wild-type mice, suggesting that homeostatic regulation of Ly6C^{hi} monocyte frequencies is CCR2 dependent. Further analysis revealed that Ly6C^{hi} monocyte frequencies were increased in the bone marrow of CCR2-deficient mice and, in the setting of *L. monocytogenes* infection, the frequency of these cells markedly increased and they upregulated MHC class II and B7.1 expression and produced TNF in the bone marrow. In contrast to Ly6C^{hi} monocytes that trafficked to the spleen in wild-type mice, Ly6C^{hi} monocytes that were retained in the bone marrow of *L. monocytogenes*-infected, CCR2-deficient mice did not express iNOS or upregulate the expression of CD11c. The reason for this disparity is unknown, but it may be the result of diminished exposure of bone marrow-retained monocytes to bacterially derived TLR ligands, which drive TipDC differentiation in the spleen. Although to a lesser extent than in CCR2-deficient mice, CCL2 deficiency also resulted in decreased emigration of Ly6C^{hi} monocytes from the bone marrow into the bloodstream (Serbina and Pamer, 2006). CCL7 also contributes to homeostatic monocyte emigration from the bone marrow. The frequency of Ly6C^{hi} monocytes in the circulation of CCL7-deficient mice is reduced by approximately 60%, while the frequency of monocytes in the bone marrow is increased. During *L. monocytogenes* infection of CCL7-deficient mice, the frequency of Ly6C^{hi} monocytes in the bone marrow remained elevated, a result of diminished emigration into the bloodstream (Jia *et al.*, 2008).

11. LY6C^{HI} MONOCYTE RECRUITMENT TO INFECTED TISSUES

Although CCR2-signaling mediates monocyte emigration from the bone marrow into the bloodstream, whether CCR2 also contributes to tissue infiltration by Ly6C^{hi} monocytes remains more controversial. To determine whether CCR2 is required for Ly6C^{hi} monocyte recruitment from the bloodstream into the spleens of *L. monocytogenes*-infected mice, blood monocytes from CCR2-deficient or CCR2-sufficient mice were transferred into infected recipient mice and their trafficking to the spleen was quantified (Serbina and Pamer, 2006). *L. monocytogenes* infection in spleen is largely restricted to the T cell zones of the white pulp (Conlan, 1996), so localization of monocytes to infected white pulp regions was also measured. These studies demonstrated that localization of CCR2-deficient and -sufficient monocytes in the spleen was similar, suggesting that CCR2 is not required for trafficking from the bloodstream into the spleen or, within the spleen, into foci of *L. monocytogenes* infection within the white pulp.

Infection of the liver is also markedly enhanced in CCR2-deficient mice (Kurihara *et al.*, 1997). Ly6C^{hi} monocytes are infrequent in uninfected livers, representing only 1–2% of CD45^{+} cells, but, upon *L. monocytogenes* infection, their frequency increases dramatically, and they represent nearly 30% of CD45^{+} cells 3 days following intravenous inoculation (Shi *et al.*, 2010). Histologic analyses demonstrated that Ly6C^{hi} monocytes localize to sites of *L. monocytogenes* infection in the liver, surrounding foci of bacteria and necrotic hepatocytes. Monocytes recruited to the liver express TNF and iNOS and upregulate surface MHC class II expression and thus are similar to splenic TipDCs. Adoptive transfer of wild-type or CCR2-deficient monocytes into *L. monocytogenes*-infected recipient mice demonstrated that they trafficked similarly to foci of infection in the liver. Intravital microscopy demonstrated that the velocity and arrest and meandering indices of CCR2-deficient and CCR2-sufficient Ly6C^{hi} monocytes were similar in the livers of infected mice, suggesting that CCR2 does not contribute to monocyte recruitment or localization during hepatic *L. monocytogenes* infection.

Transfer of wild-type or CCR2-deficient monocytes into *L. monocytogenes*-infected, CCR2-deficient recipient mice protected against hepatic infection equivalently, demonstrating that CCR2-mediated signals are not required for intravenously inoculated monocytes to enter the liver and mediate protection. Treatment of adoptively transferred monocytes with pertussis toxin, which inhibits signaling by G-protein-coupled chemokine receptors, did not alter their trafficking to foci of *L. monocytogenes* infection in the liver, suggesting that monocyte recruitment from the bloodstream is not only CCR2 independent but generally chemokine independent (Shi *et al.*, 2010). Immunohistology demonstrated that hepatic foci of *L. monocytogenes* infected are surrounded by a zone of markedly upregulated ICAM-1 expression. ICAM-1 upregulation occurs on hepatic endothelial cells. Ly6C^{hi} monocytes localize to these sites of ICAM-1 upregulation in the liver, suggesting that monocytes are trapped in these areas. In support of this hypothesis, administration of blocking antibodies specific for ICAM-1, or CD11b, which binds ICAM-1, markedly reduces accumulation of Ly6C^{hi} monocytes in the liver (Shi *et al.*, 2010). These findings, in aggregate, suggest that monocytes localize to sites of infection in the liver by associating with ICAM-1 and that chemokine-mediated signals are not required for this process.

12. MONOCYTE EMIGRATION FROM THE BONE MARROW

Although the important role for CCR2 in the emigration of monocytes from the bone marrow has been demonstrated in many different infectious disease models (Aldridge *et al.*, 2009; Bosschaerts *et al.*, 2010; Crane *et al.*, 2009; Lim *el al.*, 2011), how infection in the lung, intestine, liver, or other central organs promotes monocyte emigration from bone marrow has been unclear. One possibility is that low-grade infection of the bone marrow serves as a stimulus to promote monocyte emigration from the bone marrow, a scenario for which there is little evidence and which seems counterintuitive (i.e., dispatching inflammatory cells away from a site of infection). Alternatively, systemic infections might induce local production of chemokines that enter the circulation and trigger responses in the bone marrow. This model is plausible but would require synthesis and secretion of large quantities of chemokine. Measurement of circulating chemokine levels reveals that they are produced in large quantities, but generally during late stages of infection. A third possibility

is that the bone marrow detects low levels of circulating microbial molecules during infection and responds by expressing chemokines that promote inflammatory cell emigration into the bloodstream. This model would require the bone marrow compartment to detect circulating microbial molecules with great sensitivity, and it would require chemokines, CCL2 in the case of *L. monocytogenes* infection, to drive monocytes into the bloodstream.

To determine whether circulating microbial molecules influence bloodstream monocyte frequencies, TLR2, TLR4, TLR5, and TLR9 ligands were administered to mice and shown to markedly increase circulating Ly6C^{hi} inflammatory monocyte frequencies (Shi *et al.*, 2011). Increases in circulating monocyte frequencies in response to different TLR ligands required expression of the corresponding TLR. Thus, LPS administration did not induce monocyte emigration from the bone marrow in the absence of TLR4. Ly6C^{hi} monocyte frequencies in the bloodstream only increased when TLR ligands were administered at low concentrations. The dosage of LPS that increased the frequency of circulating monocytes ranged from 2 to 200 ng and higher doses of LPS did not increase circulating monocyte frequencies. The ineffectiveness of higher doses of LPS was surprising and may be the result of monocyte retention in the bone marrow by mechanisms that remain incompletely defined. In contrast to monocyte emigration from the bone marrow, neutrophils are recruited into the bloodstream by high doses of LPS.

Because many nucleated cells can produce CCL2 upon stimulation, it has remained unclear which CCL2-producing cells *in vivo* are driving monocyte trafficking. To determine whether CCL2 production by bone marrow-derived cell populations is required for monocyte trafficking, bone marrow chimeric mice were generated between CCL2-deficient and wild-type mice. These experiments demonstrated that CCL2 production by nonhematopoietic cells is required for monocyte emigration from the bone marrow (Shi *et al.*, 2011). This suggested that stromal cells, endothelial cells, or perhaps hepatocytes were producing CCL2 in order to drive monocyte emigration from the bone marrow into the bloodstream. To determine which cells produced CCL2 *in vivo* during *L. monocytogenes* infection, CCL2-reporter mice were generated. As a first step, BAC-transgenic mice in which the CCL2 promoter drives EGFP expression were produced. To simplify interpretation of this experiment, CCL2-EGFP reporter mice were irradiated and transplanted with wild-type bone marrow to restrict EGFP expression to nonhematopoietic cells. Infection of these mice with *L. monocytogenes*, or inoculation with low doses of LPS, demonstrated that CCL2 expression, as determined by EGFP fluorescence, was induced in stromal and endothelial cells of the bone marrow. Flow cytometric analysis revealed that the majority of CCL2-expressing cells were mesenchymal stem cells (MSCs) and/or CXCL12 abundant reticular (CAR) cells (Mendez-Ferrer *et al.*, 2010; Sugiyama *et al.*, 2006). These cells express TLR-2, -3, -4, -8, and -9 and reside in close proximity to bone marrow endothelial cells, and are also referred to as pericytes. These results suggested that MSCs and/or CAR cells respond to TLR ligands or to systemic *L. monocytogenes* infection and produce CCL2.

To determine whether CCL2 production by MSCs is required for monocyte emigration from the bone marrow, a conditional CCL2 knockout mouse strain was generated and crossed to the Nestin-Cre mouse (Shi *et al.*, 2011). Previous studies had demonstrated that bone marrow MSCs express Nestin (Mendez-Ferrer *et al.*, 2010), and thus, Nestin-Cre mice

selectively delete CCL2 from MSCs in the bone marrow. Using this system, it was demonstrated that CCL2 production by MSCs contributes to monocyte emigration in response to low-dose administration of LPS and during *L. monocytogenes* infection. Further, deletion of CCL2 in MSCs renders mice more susceptible to *L. monocytogenes* infection, with decreased bacterial clearance from the liver and spleen. This study demonstrated that a stromal cell population monitors the circulation for the presence of microbial molecules and regulates the emigration of Ly6C^{hi} monocytes from the bone marrow in the bloodstream.

13. CONCLUSIONS

The past decade has seen great progress in our understanding of monocyte-mediated immune defense against viral and microbial pathogens (Serbina *et al.*, 2008); however, many questions remain unanswered. While inflammatory monocytes and their progeny, TipDCs, play an essential role in defense against *L. monocytogenes* infection, and the absence of Ly6C^{hi} monocytes renders mice highly susceptible, it remains unclear how monocytes mediate bacterial clearance in the liver and spleen. While TNF and iNOS contribute to innate immune defense, and TipDCs are the major producers of these two proteins during early infection, it is unknown whether TNF or iNOS production by TipDCs is essential for their antimicrobial activity. Questions about monocyte emigration from the bone marrow, such as how CCL2 and CCL7 mediate Ly6C^{hi} monocyte trafficking from the abluminal to luminal side of bone marrow endothelial cells, also remain unanswered. Given the increasing number of useful mouse strains that enable intravital analyses of monocyte trafficking, it is likely that our understanding of monocytes and their role in defense against murine *L. monocytogenes* infection will continue to increase in the coming years.

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