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Severe *Listeria monocytogenes* Infection Induces Development of Monocytes with Distinct Phenotypic and Functional Features


Monocytes perform diverse roles during infection with the facultative intracellular bacterium *Listeria monocytogenes*. They are essential as bactericidal cells in host defense but can also become Trojan horses transporting bacteria into the brain. To explain these contrasting roles, we characterized bone marrow (BM) monocytes in steady state and generated during lethal and sublethal *L. monocytogenes* infection. Ly-6C<sup>hi</sup>CD11b<sup>+</sup> BM monocytes expressed high amounts of M-CSFR/CD115 in steady state and 72 h following sublethal infection. However, infection with increasing numbers of bacteria resulted in progressive loss of CD115 and strongly decreased CD115-encoding c-fms mRNA expression. Conversely, analysis of regulatory molecules showed de novo expression of the nonsignaling IL-1R<sub>i</sub>, CD121b, under the same conditions. Ly-6C<sup>hi</sup>-CD11b<sup>+</sup> monocytes in circulation also acquired a CD115<sup>neg/low</sup>CD11b<sup>hi</sup> phenotype during lethal infection. These BM monocytes showed upregulation of suppressor of cytokine signaling 1 and 3 and IL-1R–associated kinase-M to a greater extent and/or earlier compared with cells from sublethal infection and showed decreased LPS-induced IL-6 production despite similar levels of surface TLR4 expression. BM monocytes from uninfected or sublethally infected mice bound and internalized very few *L. monocytogenes* in vitro. However, both functions were significantly increased in monocytes developing during lethal infection. Nonetheless, these cells did not produce reactive oxygen intermediates, suggesting an inability to kill *L. monocytogenes*. Together, these data show that systemic infections with lethal and sublethal amounts of bacteria differentially shape developing BM monocytes. This results in distinct phenotypic and functional properties consistent with being Trojan horses rather than bactericidal effector cells.

Erie monocyte responses are carefully balanced to ensure adequate host defenses to pathogenic stimuli and, simultaneously, to prevent excess tissue damage caused by the inflammatory response (1, 2). *Listeria monocytogenes* is a model bacterial pathogen that causes systemic infections in humans and animals with predilection to invade the CNS (3). Interactions between *L. monocytogenes* and mononuclear phagocytes are an interesting in vivo paradigm of the balance between inflammation and regulation because these cells can function as bactericidal effector cells but also can be permissive for intracellular bacterial growth (4). In particular, Ly-6C<sup>hi</sup> monocytes are crucial to the pathogenesis of CNS infection by acting as Trojan horses that transport intracellular *L. monocytogenes* into the CNS (5, 6). However, these same cells are also immediate precursors for cytokine-activated macrophages and TNF-α-inducible NO synthase-producing dendritic cells required for overcoming *L. monocytogenes* infection (7, 8).

The purpose of this study was to gain insight into how Ly-6C<sup>hi</sup> BM monocytes in the blood are recent emigrants from the bone marrow (BM) (9). In the setting of *L. monocytogenes* infection, some of these cells are parasitized in the BM before entering the circulation (6). The BM is usually sterile 1–4 h after i.v. or i.p. injection of *L. monocytogenes*, but bacteria can be recovered from it 24 h postinfection (10, 11). After invading the BM, *L. monocytogenes* replicates rapidly for ~72 h, then the number of bacteria declines in sublethal infection but continues to increase in lethal infection (6, 10).

In BM of lethally infected mice, the majority of *L. monocytogenes* localize to Ly-6C<sup>hi</sup>CD11b<sup>hi</sup> monocyte precursors in which they escape from phagosomes and replicate intracellularly (6, 11). Parasitized monocytes are also found in the peripheral circulation and transport intracellular bacteria to the brain (5, 6, 12). These data indicate that BM monocytes developing during lethal infection fail to acquire bactericidal activity despite the abundant presence of IFN-γ and TNF-α, which typically induce bactericidal activity in macrophage precursor cells with a similar phenotype in steady state and sublethal infection (13).

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Abbreviations used in this paper: BM, bone marrow; DHR, dihydrorhodamine; IRAK-M, IL-1R-associated kinase-M; ROS, reactive oxygen species; SOCS, suppressor of cytokine signaling; TACE, TNF-α-converting enzyme; TAPI 2, TNF-α protease inhibitor.

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making these cells unresponsive to proinflammatory stimuli that could trigger bacterial activity.

Materials and Methods

**Abs**

Fluorochrome-conjugated mAb directed against CD11b (M1/70), CD11c, CD31, CD43, CD54, CD62L (MEL-14), CD120b, CD121b, CD126, Ly-6G (1A8), and Mac-3 (CD107b) and isotype control mAb were purchased from BD Pharmergen (San Diego, CA). F4/80-PE was purchased from Serotec (Raleigh, NC), and anti–IL-6–PE was purchased from eBiosciences (San Diego, CA). Rat anti-mouse Ly-6c (ER-MP20) (14) and rat anti-mouse TLR4/MD2 (Cell Sciences, Canton, MA) were used as direct FITC conjugates.

**Bacteria**

Wild type *L. monocytogenes* strain EGD (LD$_{50}$ ∼4.0 log10 i.v.) was stored in brain heart infusion broth (Difco, Detroit, MI) at 10$^7$ CFU/ml at −70°C. For experiments, 0.5 ml stock culture was diluted in 4 ml broth and then cultured for 4.5 h at 37°C to obtain bacteria in logarithmic growth phase. Bacteria were diluted into sterile PBS prior to injection into mice.

**Mouse infection**

Eight- to 16-wk-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. They were infected by i.v. injection of *L. monocytogenes* and were euthanized at the indicated times with ketamine/xylazine (Vedco, St. Joseph, MO). Blood was collected into PBS containing 10 mM EDTA, and blood leukocytes were isolated as described (5). BM was harvested from the tibia and femur by dissecting away soft tissues and cutting bones into smaller sections prior to flushing them with a pestle in sterile six-well dishes in 2 ml DMEM containing 0.5% BSA and 10 mM EDTA. The wells were washed to collect the cells, which were then passed through a 100-μm filter before centrifugation. Erythrocytes were lysed at room temperature for 8 min in 5 ml diluted Pharm-lyse (BD Pharmingen) and then the leukocytes were washed three times with DMEM containing 0.5% BSA and 10 mM EDTA and counted in a hemacytometer. Animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center and the Oklahoma City VA Medical Center.

**IL-6 production**

BM leukocytes were cultured in a humidified incubator at 37°C with 5% CO$_2$ at a density of 10$^6$ cells/ml in DMEM containing 5% FCS plus penicillin G (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated overnight without or with LPS at 10 or 100 ng/ml. Supernatants were removed, centrifuged to remove detached cells, and the concentration of IL-6 was measured by ELISA (R&D Systems, Minneapolis, MN).

**Flow cytometry and cell sorting**

Samples of 10$^5$ leukocytes were incubated in 96-well microtiter plates with 3% normal mouse serum and anti-CD16/32 mAb (BD Pharmingen) for 30 min on ice prior to the addition of isotype-matched control or test mAb. Cells were incubated with mAb for 30 min and then were washed three times with PBS/BSA/azide and postfixed with 1% paraformaldehyde. For intracellular CD115 staining, cells were incubated with Ly-6c and CD11b mAb, fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and incubated with CD115 mAb. For intracellular IL-6 staining, 1×10$^5$ BM cells from each mouse were seeded in sterile six-well culture plates in DMEM/10% FBS with penicillin (100 U/ml) and streptomycin (100 μg/ml) and incubated in the absence or presence of LPS (10 and 100 μg/ml) overnight at 5% CO$_2$. Cells were incubated in brefeldin A (eBioscience) for 6 h prior to flow-cytometric analysis. Cells were fixed with IFN-γ Fixation Buffer (eBioscience), washed twice with permeabilization buffer (eBioscience), and stained for IL-6, according to the manufacturer’s protocol. Cells were analyzed on a FACSCalibur (BD Pharmingen). Production of reactive oxygen species (ROS) was assessed by flow cytometry by incubating cells in vitro with 15 μM dihydrorhodamine (DHR) 123 (Invitrogen, Carlsbad, CA) followed by analysis by flow cytometry (15).

**Phagocytosis assay**

Two to 3×10$^5$ BM monocytes (Ly-6c$^-$CD11b$^+$) were isolated by flow cytometry-based sorting on a MoStar (DakoCytomation, Fort Collins, CO) from steady-state mice or from animals infected 72 h earlier with sublethal (2.2–2.5 log10) or lethal (4.1–4.3 log10) amounts of *L. monocytogenes*. The cells were mixed 1:15 with culture-grown *L. monocytogenes* in DMEM plus 10% v/v normal mouse serum for 30 min at 37°C with rotation giving.

The cells were washed and cytocentrifuged onto coverslips and then fixed with 2% paraformaldehyde for 10 min at room temperature. Bacteria were identified by immunolabeling with rabbit anti-*L. monocytogenes* (Difco) antisera and CY-2 (green)-labeled anti-rabbit Ig secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Next, cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and stained with anti–Listeria antisera and Texas Red-labeled secondary Ab (Jackson ImmunoResearch Laboratories). Nuclear counterstaining was performed with DAPI (Molecular Probes, Eugene, OR). Cells and bacteria were quantified by fluorescence microscopy under oil immersion (∼1000 magnification). Bacteria bound to the outside of the cell displayed green and red fluorescence, whereas intracellular bacteria fluoresced red only.

**Quantitative real-time PCR**

RNA was extracted from BM cells using a standard protocol applying TRIzol LS reagent (Invitrogen), according to the manufacturer’s instructions, followed by genomic DNA digestion with DNaseff (Ambion, Austin, TX). Total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR reactions were run at 25-µl volumes in 96-well optical reaction plates using the Stratagene MX3005P system. Reverse-transcribed cDNA and reverse-transcribed negative controls were diluted to 5 ng/µl and then real-time PCR reactions were run with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Custom-made primers from IDT Technologies (Coralville, IA) were designed using Beacon Designer 4.02 Software (Premier Biosoft International, Palo Alto, CA) and run using optimized primer concentrations (Supplemental Table I). Thermocycling conditions were as follows: hold at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, hold at 95°C for 15 s, hold at 60°C for 20 s, ramp to 95°C for a dissociation curve, followed by a hold at 95°C for 15 s. Data were analyzed using the standard curve method.

**Statistical analysis**

Differences between groups were analyzed with the two-tailed Student t test with equal variance, with a level of significance set at $p < 0.05$.

**Results**

*L. monocytogenes* infection induces loss of CD115 (M-CSFR) expression from BM monocytes

To analyze mononuclear phagocyte development during *L. monocytogenes* infection, we used M-CSFR/CD115 in combination with other markers of the lineage to identify these cells. At steady state, 13.8 ± 0.5% (mean ± SEM) (n = 9) of total BM cells expressed high levels of CD115 (Fig. 1A). Microscopic analysis of sorted CD115high cells from normal mouse BM showed that 70% had characteristic monocytic morphology with indented nucleus and abundant basophilic to neutrophilic cytoplasm, with most of the remaining cells appearing less mature (data not shown). CD115$^+$ cells were predominantly Ly-6c$^-$high and showed heterogeneous expression of CD31 (Fig. 1A). In addition, they were CD11b$^+$ (80.4 ± 2.5%), and uniformly Ly-6c$^{neg/low}$ (data not shown). Sublethal *L. monocytogenes* infection with 3.3 log10 CFU bacteria induced a shift in the BM, causing the prevailing phenotype of CD115$^{high}$ cells at 72 h postinfection to be Ly-6c$^{high}$ CD31$^+$, in accordance with a previously observed stimulation of myelopoiesis and increase in precursor cells (10). Infection with 2.4 log10 CFU *L. monocytogenes*, ∼0.02–0.03 LD$_{50}$ for this strain, caused a significant increase in the percentage of CD115$^+$ cells and Ly-6c$^{low/medium}$CD11b$^+$ monocytes when analyzed after 72 h, but there was no change in the percentage of Ly-6c$^{high}$CD11b$^+$ monocytes that expressed CD115 at high levels (Table I).

Interestingly, we found that lethal infection with ≥4.0 log10 CFU *L. monocytogenes* markedly decreased detection of CD115 among Ly-6c$^{low/medium}$CD11b$^+$ monocytes 72 h postinfection (Fig. 1B). Intracellular staining confirmed loss of the CD115$^{high}$ population, indicating that ligand-mediated receptor internalization did not explain the lack of CD115 detection (Fig. 1C). Despite the loss of CD115, the Ly-6c$^{high}$ cells were monocyctic; we showed previously that these cells are CD11b$^+$ and display monocytic morphology but...
do not express CD3, CD19, Ly-6G, NK1.1, or TER-119 (11). Additionally, there was no change in numbers of Ly-6C high CD11b+ BM cells from steady state (3.1 ± 1.7 × 10^5 cells/tibia+femur) compared with 72 h postinfection with the indicated amount of *L. monocytogenes*. Gates on Ly-6C^high^CD11b^+^ monocytes are shown. BM cells from mice at steady state or 72 h following infection with 4.3 log10 CFU bacteria were fixed only (upper panels) or were fixed and permeabilized (lower panels) prior to labeling with CD115 mAb. D, Representative graphs show surface CD115 expression (open) compared with isotype control mAb (shaded) on gated monocytes. The percentage of CD115^+^ cells is given. E, mRNA was extracted from flow-sorted Ly-6C^high^CD11b^+^Ly-6G^neg/low^ BM monocytes pooled from steady-state mice or from mice infected 72 h earlier with 4.0 log10 CFU *L. monocytogenes*. Expression of CD115-encoding *c-fms* mRNA was determined by quantitative PCR and normalized to β-actin.

In addition to decreased de novo gene expression, a putative explanation for loss of surface CD115 is through cleavage by TNF-α–converting enzyme (TACE) (18). To model this in vitro, BM from normal mice was incubated with and without 100 μM of the TACE inhibitor TNF-α protease inhibitor (TAPI 2) at 37˚C or maintained on ice. Surface CD115 and CD62L, also a TACE-sensitive ligand, were measured by flow cytometry (Fig. 2A, 2B). The frequency of monocytes expressing CD115 and CD62L decreased markedly at 37˚C, from 78.3 to 2.5% and from 98.3 to 18.7%, respectively. TAPI 2 treatment prevented the loss of both markers under these conditions. These results indicate that TACE activity causes simultaneous shedding of multiple surface markers. In contrast, in vivo experiments showed that surface CD54 and CD62L, both TACE targets, changed
little on BM monocytes during severe infection (Fig. 2C). These data suggest that indiscriminant TACE-mediated shedding is not the main mechanism reducing CD115 expression on BM monocytes.

**Lethal and sublethal L. monocytogenes infections differentially induce regulatory molecules in BM monocytes**

The above findings prompted us to test the extent to which lethal and sublethal infection differentially induced other key monocyte molecules. Functionally important candidates include molecules that downregulate inflammatory responses. These experiments revealed dramatic upregulation of the nonsignaling receptor for IL-1, CD121b (IL-1RII), another TACE-sensitive Ag, on monocytes after lethal infection (Fig. 3A). Dose–response experiments showed CD121b was consistently expressed at high amounts on monocytes 72 h following infection with $\pm 3.5$ log10 CFU *L. monocytogenes* but not following infection with $\leq 3.3$ log10 CFU *L. monocytogenes* (data not shown). In contrast, neutrophils expressed CD121b at steady state, and it was progressively upregulated on neutrophils taken from mice infected with increasing CFU bacteria. Experiments using CD11b-sorted BM cells showed CD121b was upregulated at the mRNA level as early as 48 h following lethal infection with 4.1 log10 CFU bacteria (Fig. 3B). Analysis of BM monocytes 5 d after sublethal infection showed upregulation of surface CD121b, although to a lesser extent than found in lethal infection. In addition, CD115 expression was reduced compared with steady state to a level similar to that found 72 h following infection with the same inoculum (Fig. 3C).

One explanation for the detection of increased amounts of CD115<sup>neg/low</sup>CD11b<sup>+</sup> monocytes in the BM is that these cells do not enter the peripheral circulation. To test this, expression of surface CD115 and CD121b was analyzed on peripheral blood and BM leukocytes from mice 72 h postinfection. As expected, blood monocytes from mice infected with low doses of bacteria were essentially uniformly positive for CD115 and negative for CD11b (Fig. 4, data not shown). However, loss of CD115 and upregulation of CD121b on Ly-6C<sup>high</sup>CD11b<sup>+</sup> blood monocytes were clearly evident following infection with greater amounts of bacteria. Analysis of paired specimens from the same animals suggested that loss of CD115 was more pronounced on BM monocytes, whereas CD121b was expressed at higher levels on blood monocytes (data not shown).

Next, we analyzed expression of intracellular regulatory molecules, such as suppressor of cytokine signaling (SOCS) 1 and 3 and IL-1R–associated kinase-M (IRAK-M) (1, 19). Data in Fig. 5A show that expression of SOCS1 by BM CD11b<sup>+</sup> myeloid cells peaked at 24 h postinfection in cells from lethal infection, whereas it peaked at 48 h postinfection in sublethal infection. SOCS3 was significantly upregulated as early as 24 h postinfection and remained significantly higher than steady state during lethal infection, whereas upregulation during sublethal infection only occurred at 48 h postinfection. In addition, upregulation of SOCS1 and SOCS3 specifically in BM monocytes was confirmed using sorted Ly-6C<sup>high</sup>CD11b<sup>+</sup> monocytes. B, Graphs show expression of CD115 and CD62L on gated Ly-6C<sup>high</sup>CD11b<sup>+</sup> monocytes shown in A and are representative of two experiments with similar results. Percentage of positive cells, compared with isotype controls, are given. C, BM cells from steady-state mice or mice infected 72 h earlier with sublethal (3.3 log10 CFU) or lethal (4.3 log10 CFU) amounts of *L. monocytogenes* were labeled with mAb against TACE targets CD54 and CD62L (open) or isotype control (shaded) and analyzed by flow cytometry. Percentages of positive cells are given.

**FIGURE 2.** Decreased CD115 expression on BM monocytes in *L. monocytogenes* infection is not due to TACE activity. BM cells from steady-state mice were held on ice or incubated for 30 min at 37°C with and without 100 μM TAPI2 (TACE inhibitor) and then were labeled with the indicated mAb and analyzed by flow cytometry. A, Dot plots show gating on Ly-6C<sup>high</sup>CD11b<sup>+</sup> monocytes. B, Graphs show expression of CD115 and CD62L on gated Ly-6C<sup>high</sup>CD11b<sup>+</sup> monocytes shown in A and are representative of two experiments with similar results. Percentage of positive cells, compared with isotype controls, are given. C, BM cells from steady-state mice or mice infected 72 h earlier with sublethal (3.3 log10 CFU) or lethal (4.3 log10 CFU) amounts of *L. monocytogenes* were labeled with mAb against TACE targets CD54 and CD62L (open) or isotype control (shaded) and analyzed by flow cytometry. Percentages of positive cells are given.
altered responses to TLR stimulation using LPS for a model TLR4 agonist. Unfractionated BM cells from uninfected mice and from mice infected 72 h earlier with lethal and sublethal amounts of *L. monocytogenes* were incubated 18 h in vitro in medium containing antibiotics plus 0, 10, or 100 ng/ml LPS. IL-6 was measured in the cell supernatants by ELISA, and intracellular IL-6 was quantified in specific populations of cells by flow cytometry. Data in Fig. 6A show that LPS stimulated IL-6 production in each group of cells and that cells from steady-state and sublethally infected mice responded the same. However, cells from lethal infection produced significantly less IL-6 in response to LPS than did cells from steady state or sublethal infection. As expected, flow cytometric analysis of intracellular IL-6 in BM cells showed that increasing amounts of infection increased the frequency of IL-6+ monocytes and neutrophils, consistent with increased systemic levels of IL-6 under the same conditions (Fig. 6B) (20). Incubation in vitro in LPS significantly increases the percentage of IL-6+ monocytes from steady-state and sublethally infected animals, but it did not change IL-6 expression in neutrophils or lymphocytes (data not shown) (Fig. 6C). In contrast, although monocytes from lethally infected animals expressed IL-6 at baseline, additional LPS stimulation actually decreased the percentage of IL-6+ cells. Flow cytometry showed that loss of TLR4 expression on Ly-6ChighCD11b+ monocytes did not explain decreased LPS-induced cytokine production (Fig. 6D).

Data indicate that severe infection can downregulate expression of CX3CR1 and CCR2 on human monocytes, changes that could impact their migratory function (21, 22). Thus, we investigated the extent to which *L. monocytogenes* infection changed the expression of mRNA for chemokine receptors in BM monocytes. Consistent with data from human monocytes, lethal *L. monocytogenes* infection downregulated CCR2 mRNA expression by 2.5–5.0-fold, but it remained the most abundant chemokine receptor expressed, which is typical for Ly-6C<sup>high</sup> monocytes (Fig. 7). However, CXCR3 and CX3CR1 were more strongly downregulated by 100-fold. In contrast, CCR1 and CCR5 were upregulated by 2.5–4.7- and 2.0–10.0-fold, respectively. Sublethal infection induced changes to the chemokine receptor mRNA expression profile intermediate between steady state and lethal infection (data not shown). Together, these results suggest that *L. monocytogenes*-induced monocytes may have a diminished capacity to respond to CX3CL1 and the IFN-induced chemokines CXCL9, -10, and -11, although responses to other inflammatory chemokines (e.g., CCL2, CCL3, CCL4, and CCL5) may be retained or increased.
Because monocytes functioning as Trojan horses in *L. monocytogenes* infection are parasitized in the BM, we tested the degree to which infection-induced monocytes had altered binding and internalization of *L. monocytogenes*. Sorted Ly-6C<sup>hi</sup>CD11b<sup>+</sup> monocytes from steady-state and infected animals were incubated with bacteria in vitro, and extracellular and intracellular bacteria were quantified by fluorescence microscopy (23). Monocytes from steady-state and sublethally infected animals bound few bacteria and internalized almost none (Table II). In contrast, monocytes from lethally infected mice bound and internalized significant numbers of bacteria. Thus, monocytes from lethal infection were associated with 3.8- and 8.1-fold more *L. monocytogenes* compared with cells from steady state and sublethal infections, respectively. To control for bacteria present in BM cells as a result of in vivo infection, duplicate samples of cells were labeled with mAb and then analyzed by fluorescence microscopy without added bacteria (Table II). These results showed that only 1.6% of BM monocytes from lethally infected mice were associated with bacteria, nearly all of which were intracellular. In contrast, no cell-associated bacteria were identified in monocytes isolated from sublethally infected mice.

Monocyte listeristic activity depends primarily on their capacity to produce reactive nitrogen and oxygen intermediates (24). Therefore, we assessed the expression of *iNOS* mRNA in total BM cells. *iNOS* expression increased progressively with increased numbers of infecting bacteria (Fig. 8A), likely in response to greater cytokine (e.g., IFN-γ and TNF-α) and TLR stimulation (20, 25, 26). However, *Arg1* mRNA expression also increased progressively in these cells, suggesting an increasing limitation to use arginine for NO production (27). Further experiments with sorted populations confirmed that these genes were upregulated in myeloid cells. Additionally, production of ROS was assessed by flow cytometry (Fig. 8B). Interestingly, ROS were barely detected in BM monocytes, but splenic Ly-6<sup>hi</sup>CD11b<sup>+</sup> monocytes from lethally and sublethally infected mice displayed a progressive increase in ROS-producing (DHR123<sup>+</sup>) cells. These data demonstrate that bacterial-killing mechanisms develop during lethal infection but are only partially expressed in BM monocytes.

**Discussion**

Infection-induced immunosuppression is an important cause of poor patient outcomes (28–30). Data presented in this article show that *L. monocytogenes* infection leads to a time- and dose-dependent induction of regulatory molecules in Ly-6<sup>hi</sup>CD11b<sup>+</sup> BM monocytes, where lethal infection elicits more rapid expression and/or excess expression of these molecules compared with sublethal infection. They are expressed in a hierarchy in terms of kinetics and the numbers of bacteria required for eliciting them. The attenuators of cytokine signaling, SOCS1 and SOCS3, are upregulated earliest and by the lowest inocula. Induction of CD121b, the type II IL-1 decoy receptor, requires infection with ~10-fold more bacteria and occurs 24 h later. Last induced is the negative regulator of TLR signaling IRAK-M, which is only upregulated following the highest inocula, again ~10-fold more than required for CD121b induction. Importantly, lethal, but not sublethal, infection also stimulates BM monocytes to acquire phagocytic activity without concomitant acquisition of bacterial mechanisms (e.g., production of reactive oxygen intermediates).

Thus, under these conditions, BM monocytes are more likely to become Trojan horses rather than cytokine-activated killers of intracellular bacteria.

The innate immune response increases in vigor and intensity with greater numbers of infecting *L. monocytogenes*. This is manifested, in part, by increased levels of proinflammatory cytokines and mediators, such as reactive nitrogen intermediates, as well as by increased expression of anti-inflammatory molecules and mechanisms (20, 25, 31). Within the first 24 h of infection, *L. monocytogenes* elicits high systemic levels of type I and II IFNs, as well as IL-1, IL-6, and TNF-α, which peak later (32, 33). These systemic mediators and the innate signaling pathways that trigger them induce subsequent expression of regulatory molecules that limit the extent of inflammation. For example, SOCS1 and SOCS3 are upregulated by signaling pathways activated by these cytokines in addition to TLR signaling induced by bacterial components (34, 35). Interestingly, in vitro *L. monocytogenes* infection of BM-derived macrophages and macrophage cell lines upregulates SOCS1 and SOCS3 within 4 h (36, 37). However, these studies used multiplicities of infection of 5–10 bacteria per cell, whereas BM contains only ~2 logs of bacteria per 10<sup>6</sup> cells 24 h postinfection, with 4.3 log10 CFU *L. monocytogenes* in vivo (11). Moreover, data presented in this article show that <5% of monocytes contain intracellular bacteria 72 h postinfection. These results suggest that systemic cytokine stimulation, rather than intracellular infection, provides the initial stimuli for upregulating SOCS1 and SOCS3 in vivo, although parasitism of cells may become more important as the infection progresses.

The glucocorticoid, corticosterone, is an anti-inflammatory hormone present in elevated levels in plasma of *L. monocytogenes*-infected mice. During infection, corticosterone is produced by the adrenal gland in response to cytokine release and TLR stimulation. By binding to the glucocorticoid receptor (GR), corticosterone inhibits the expression of inflammatory genes, including *iNOS* and *Arg1*. This inhibitory effect is mediated by GR-dependent and -independent mechanisms. GR-dependent mechanisms involve the repression of gene transcription by interacting with the GR and co-repressors in the nucleus. GR-independent mechanisms involve the repression of gene transcription by interacting with the GR at the plasma membrane.
infected mice that increases proportionately with increasing bacterial load (31). Glucocorticoids induce upregulation of CD11b in macrophages, whereas intracellular L. monocytogenes infection does not, again demonstrating a key role for systemic mediators (38–40). Glucocorticoids also induce phagocytic activity in monocytes (39). This is relevant because previous studies suggest that the absence of phagocytic activity is an effective defense mechanism of hematopoietic stem cells against bacteria capable of intracellular parasitism (41). We found that BM monocytes from steady-state mice bind very few L. monocytogenes and internalize ≤5% of bound bacteria. Interestingly, the inflammatory milieu associated with sublethal infection did not induce phagocytic activity, whereas lethal infection did. The combination of induced phagocytic activity without the ability to generate reactive oxygen intermediates is particularly relevant for the development of cellular parasitism (41). We found that BM monocytes from steady-state mice are permissive to intracellular parasitism by L. monocytogenes and are loaded with bacteria in the BM (6, 11).

IRAK-M is another regulatory factor induced in developing BM monocytes during severe L. monocytogenes infection. It is specific to cells of the monocytic lineage and is upregulated by a variety of TLR agonists, as well as other means (e.g., TNF-α) (42–45). Induction of IRAK-M was only found 72 h postinfection with the largest amount of L. monocytogenes. In this respect, it is interesting to note that high concentrations of peptidoglycan are required for inducing IRAK-M expression via TLR2 in a macrophage cell line (45). Thus, it is possible that upregulation of IRAK-M during L. monocytogenes infection in vivo is confined to infected cells, although intracellular infection is not required in other infection models (46). Given the abundance of mechanisms used for regulating inflammatory responses, it is likely that others are engaged in addition to those identified in this study (1).

The regulatory molecules inhibit inflammatory responses and host defenses on multiple levels. CD11b is a cell-bound, nonsignaling “decoy” receptor that acts as a sink for soluble IL-1α and β (47). SOCS1 and SOCS3 reduce signaling via cytokine receptors by inhibiting JAK/STAT signaling (34, 35). IRAK-M inhibits TLR and IL-1R signaling in macrophages by acting as an alternatively spliced nonsignaling variant of the signaling IL-1R-associated kinase (44). Although our experiments did not directly analyze their individual impact on host defenses against L. monocytogenes infection, data suggest their combined contributions are notable. For example, interruption of IL-1 binding to the type 1 IL-1R by genetic deletion of the receptor, by administration of Ab directed against the type 1 IL-1R, or by genetic overproduction of IL-1R antagonist each render mice more susceptible to L. monocytogenes infection (48, 49). In vitro experiments showed that IRAK-M–/– macrophages infected by L. monocytogenes produced greater amounts of IL-6 and IL12 p40 than did wild type cells, but experiments analyzing L. monocytogenes infection of IRAK-M–/– mice were not performed (44). Interestingly, deletion of IL-1R–associated kinase, a putative target for IRAK-M, did not impair the ability of mice to eliminate L. monocytogenes, suggesting that upregulation of IRAK-M alone may not be sufficient to alter susceptibility in vivo.
cells pooled from two to four mice per group.

Importantly, SOCS1 and/or SOCS3 are upregulated in infection with 4.3 (Experiment 1) or 4.1 (Experiment 2) log10 CFU of L. monocytogenes, and then isolated mRNA was analyzed by quantitative PCR for chemokine receptor expression. Results are presented as the normalized mRNA expression (gene/β-actin) of the indicated receptor in cells pooled from two to four mice per group.

FIGURE 7. L. monocytogenes infection alters BM monocyte chemokine receptor expression. Ly-6C<sup>high</sup>CD11b<sup>+</sup> BM monocytes were sorted by flow cytometry from normal mice at steady state or 72 h following lethal infection with 4.3 (Experiment 1) or 4.1 (Experiment 2) log10 CFU of L. monocytogenes and then isolated mRNA was analyzed by quantitative PCR for chemokine receptor expression. Results are presented as the normalized mRNA expression (gene/β-actin) of the indicated receptor in cells pooled from two to four mice per group.

(50). Nonetheless, upregulation of IRAK-M is associated with death during pediatric multiple organ dysfunction syndrome and severe *Burkholderia pseudomallei* infection, suggesting it has a key role in infection-induced immunosuppression in vivo (29, 30).

SOCS1 and SOCS3 are required for normal homeostasis, and mice genetically deficient in them exhibit neonatal or embryonic lethality, respectively, in contrast to IRAK-M<sup>−/−</sup> mice, which develop normally (44, 51). Importantly, SOCS1 and/or SOCS3 are upregulated in macrophages by several intracellular pathogens, including *L. monocytogenes*, *Toxoplasma gondii*, *Leishmania major*, and *Mycobacterium avium*, and inhibit the ability of IFN-γ to activate macrophages and kill intracellular organisms (37, 52–54). Paradoxically, however, SOCS3 also seems to be required for macrophages to become classically activated (55). Thus, the levels of these molecules likely determine their functional impact, and large amounts in BM monocytes probably affect killing of *L. monocytogenes* negatively. The critical roles of SOCS1 and SOCS3 in normal homeostasis are consistent with their upregulation with the lowest inocula of *L. monocytogenes*. Similarly, CD121b is upregulated by 72 h of sublethal infection on neutrophils and blood monocytes, as well as on BM monocytes 120 h after sublethal infection, when bacterial counts are rapidly declining. Expression of IRAK-M was not evaluated at time points >72 h. Thus, it is not clear whether it is also upregulated in sublethal *L. monocytogenes* infection or is specific to lethal infection.

Another key finding of our experiments is a dose-dependent reduction of CD115/M-CSF receptor expression by Ly-6C<sup>high</sup>CD11b<sup>+</sup> BM monocytes developing during infection. Nonetheless, these cells remain in the monocyte lineage, as shown by lack of the granulocytic-specific marker Ly-6G or other lineage markers and preserved monocytic morphology (11). These changes are not simply explained by different maturational stages because cells from lethal and sublethal infections display similar expression of the developmental markers Ly-6C and CD31 (10). Moreover, we found that some degree of CD115 downregulation was also present in sublethal infection acutely, as well as 120 h postinfection. Loss of surface CD115 is triggered by a variety of stimuli and processes, such as internalization of M-CSF–CD115 complexes and TACE-mediated shedding during macrophage activation by IFN-γ/IFN-γR, TNF-α, and bacterial CpG-DNA (18, 56–59). However, the absence of significant levels of intracellular M-CSFR makes it implausible that ligand-mediated receptor internalization explains the paucity of surface expression. Additionally, preserved or de novo expression of TACE-sensitive markers, such as CD54, CD62L, and CD121b, suggest that TACE-mediated shedding is an inadequate explanation. Nevertheless, it is possible that these ligands have differential sensitivity to sheddases or that different mechanisms for CD115 downregulation are engaged in sublethal and lethal infections (60). Although we cannot fully exclude that CD115<sup>−/−</sup> BM monocytes are selectively killed during *L. monocytogenes* infection (e.g., by having greater phagocytic capability than CD115<sup>+</sup> BM monocytes), we found no quantitative evidence of widespread elimination of Ly-6C<sup>high</sup>CD11b<sup>+</sup> BM monocytes. Therefore, we regard the

Table II. Sublethal infection increases binding and internalization of *L. monocytogenes* by Ly-6C<sup>high</sup>CD11b<sup>+</sup> BM monocytes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Monocytes Associated with Bacteria (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bacteria/ Monocyte</th>
<th>Intracellular Bacteria (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state</td>
<td>5.31 ± 1.76</td>
<td>1.54 ± 0.54</td>
<td>4.0 ± 2.4</td>
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<tr>
<td>Sublethal</td>
<td>4.04 ± 2.01</td>
<td>1.01 ± 0.03</td>
<td>1.0 ± 1.3</td>
</tr>
<tr>
<td>Lethal</td>
<td>10.19 ± 1.77*</td>
<td>3.29 ± 0.68*</td>
<td>25.2 ± 1.6*</td>
</tr>
<tr>
<td>Lethal control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.60 ± 1.15</td>
<td>1.94 ± 0.92</td>
<td>96.4 ± 3.6*</td>
</tr>
</tbody>
</table>

Ly-6C<sup>high</sup>CD11b<sup>+</sup> BM monocytes were sorted by flow cytometry from steady-state animals or from animals infected 72 h earlier with sublethal (2.5 log10 CFU) or lethal (4.3 log10 CFU) amounts of *L. monocytogenes*. Cells and bacteria were mixed 1:15 in the presence of 10% normal mouse serum. Intracellular bacteria and extracellular bacteria bound to cells were differentially labeled and then quantified by fluorescence microscopy.

<sup>a</sup>Results presented are the mean ± SEM from 4–8 individual mice per group.

<sup>b</sup>Calculated as (intracellular bacteria/total bacteria) × 100.

*No bacteria were added to cells from lethally infected mice.

*<i>p</i> < 0.02 compared with steady state; two-tailed Student t test.

FIGURE 8. Increased bacterial loads stimulate inducible NO synthase and Arg1 expression but not ROS production in BM cells. A. Expressions of mRNA for iNOS and Arg1 were measured in total BM cells from steady-state mice or from mice euthanized 72 h following infection with the indicated amounts of bacteria. Results are the mean normalized expression (gene/β-actin) from three or four mice per group. *p < 0.05, compared with steady state.

B. BM and spleen cells were harvested from steady-state mice or mice infected 72 h earlier with sublethal (2.2 log10 CFU) or lethal (4.3 log10 CFU) amounts of *L. monocytogenes*. Cells were incubated in vitro with 15 μM DHR123 and analyzed by flow cytometry. Graphs show representative results from gated Ly-6C<sup>high</sup>CD11b<sup>+</sup> monocytes obtained from steady-state (thick broken line), sublethal (solid line, no shading), and lethal (solid line, shaded) groups.
observed downregulation of CD115/M-CSFR mRNA levels in developing monocytes, by transcriptional and/or posttranscriptional mechanisms, the most likely explanation for the appearance of M-CSFR^{low} monocytes in *L. monocytogenes* infection (61). In accordance, several inflammatory mediators have been shown to decrease transcription of M-CSFR (62, 63), whereas microRNAs targeting M-CSFR mRNA for degradation might also be upregulated during *L. monocytogenes* infection (64).

What could be the impact of the loss of M-CSF responsiveness by cells of the monocytic lineage developing in *L. monocytogenes* infection? First of all, M-CSF is unlikely to be the driving force for continued development of these cells. This is despite the fact that M-CSF is the most specific and well-characterized growth factor known for the monocytic lineage, and recent findings suggest that *L. monocytogenes* infection rather selectively induces monoipoiesis (65). In this situation, increased myelopoiesis could be driven by other CSFs targeting the myelomonocytic lineage, such as GM-CSF and G-CSF, which are induced at high levels during infection (66, 67). Interestingly, SOCS3 was reported to suppress G-CSF–mediated emergency granulopoiesis and may, therefore, contribute to the selective expansion of monocytic cells from myeloid progenitors by factors other than M-CSF (68). The loss of surface M-CSFR/CD115 may reconcile apparently disparate data showing that *L. monocytogenes* infection stimulated a strong increase of monopoiesis (10, 65) and early findings by other investigators that *L. monocytogenes* infection caused a strong decrease in the number of M-CSF–binding and -responsive BM cells (69, 70). Furthermore, loss of M-CSF responsiveness also impacts the functional activation of monocytic cells. For example, M-CSF stimulation of isolated macrophages enhances intracellular *L. monocytogenes* growth (71). Similarly, BM-derived macrophages generated in vitro through M-CSF stimulation were permissive for intracellular bacterial growth (72) or killed significantly less *L. monocytogenes* than GM-CSF-stimulated BM-derived macrophages (73). These results are consistent with identification of an alternative (M2-like) activation signature in M-CSF–stimulated macrophages, whereas GM-CSF–stimulated macrophages seem primed to develop an M1-like classical activation signature (74). Thus, decreasing cellular sensitivity to M-CSF during *L. monocytogenes* infection could function to optimize innate defenses because a polarized M2-type response would be detrimental.

Innate immune responses are tightly controlled to enable downregulation of appropriate inflammatory responses following elimination of pathogens and to prevent excessive inflammatory responses to nonpathogenic stimuli (1, 2). In some situations (e.g., the sepsis syndrome), this balance is disturbed by extreme proinflammatory stimuli, with the result that suppressive responses predominate over proinflammatory responses to the detriment of the host (75). At the level of the monocyte, this is accompanied by dysregulation and unresponsiveness to second stimuli (29, 30, 46, 76). Our results indicate that lethal *L. monocytogenes* infection is manifested, in part, by a failure of host defenses at the level of the developing Ly-6C^{high} monocyte. Data presented in this article suggest that excessive negative regulation is a key factor that leads to monocytic paralysis and the subsequent inability to cope with the pathogen burden. Furthermore, these results identify a novel mechanism by which intracellular bacteria subvert host defenses by skewing developing monocytes toward becoming Trojan horses.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


**Supplemental Table 1**  Primer pairs used for quantitative real-time PCR.

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<th>Target</th>
<th>Primer sequence</th>
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</tr>
<tr>
<td></td>
<td>anti-sense 5'-CTGTGTTCCTCCAGAGATT-3'</td>
</tr>
<tr>
<td>CCR2</td>
<td>sense 5'-GGAGCCATACCTGTAAATGC-3'</td>
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<td></td>
<td>anti-sense 5'-TTGTCTTCCATTTCCCTTGATTTG-3'</td>
</tr>
<tr>
<td>CCR5</td>
<td>sense 5'-AGCCAGAGGAGGTGAGATCAC-3'</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>CXCR3</td>
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<td>anti-sense 5'-TCGCCCAAATAACAGG-3'</td>
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