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Monocyte Trafficking to Hepatic Sites of Bacterial Infection Is Chemokine Independent and Directed by Focal Intercellular Adhesion Molecule-1 Expression

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Recruitment of CCR2+Ly6C<sup>hi</sup> monocytes to sites of infection is essential for efficient clearance of microbial pathogens. Although CCR2-mediated signals promote monocyte emigration from bone marrow, the contribution of CCR2 to later stages of monocyte recruitment remains unresolved. In this article, we show that CCR2 deficiency markedly worsens hepatic Listeria monocytogenes infection because Ly6C<sup>hi</sup> monocytes are retained in the bone marrow. Intravenously transferred, CCR2-deficient Ly6C<sup>hi</sup> monocytes traffic normally to hepatic foci of infection and contribute to bacterial clearance. Pertussis toxin treatment of adaptively transferred monocytes does not impair their intrahepatic trafficking, suggesting that chemokine signaling, once CCR2+Ly6C<sup>hi</sup> monocytes emigrate from the bone marrow, is not required for monocyte localization to sites of bacterial infection in the liver. Expression of ICAM-1 is induced in close proximity to foci of bacterial infection in the liver, including on CD31<sup>+</sup> endothelial cells, and blockade of CD11b and CD44 diminishes monocyte localization to these hepatic foci. Our studies demonstrated that Ly6C<sup>hi</sup> monocyte recruitment from the bloodstream to the L. monocytogenes-infected liver does not require chemokine receptor-mediated signals but instead is principally dependent on integrin- and extracellular matrix-mediated monocyte adhesion. The Journal of Immunology, 2010, 184: 6266–6274.

Monocytes are myeloid leukocytes that reside in the bloodstream, bone marrow, and spleen. Murine blood monocytes are divided into two major subsets based on their differential expression of surface markers and trafficking behaviors. One subset expresses high levels of the chemokine receptor CX3CR1 and patrols the luminal surface of blood vessels. The other subset, also referred to as inflammatory monocytes, expresses lower levels of CX3CR1 and high levels of CCR2 and Ly6C and is recruited to sites of inflammation (1). Inflammatory Ly6C<sup>hi</sup> monocytes can differentiate into mucosal dendritic cells (DCs) after their depletion from gut and lung (2–5) and contribute to inflammatory diseases, such as atherosclerosis and multiple sclerosis (6, 7). Ly6C<sup>hi</sup> monocytes make essential contributions to immune defense against microbial pathogens, including Listeria monocytogenes (8, 9), Toxoplasma gondii (10, 11), Brucella melitensis (12), and Mycobacterium tuberculosis (13).

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Abbreviations used in this paper: CMTMR, 5-((4-chloromethyl)benzoyl)aminotetramethylrhodamine; DC, dendritic cell; EGFP, enhanced GFP; HA, hyaluronan; iNOS, inducible NO synthase; TipDC, TNF- and inducible NO synthase-producing dendritic cell.

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L. monocytogenes, a Gram-positive bacterium, infects humans via the gastrointestinal tract (14) and is recognized by the innate immune system with TLR- and Nod-like receptor-mediated mechanisms (15–18). Intravenous inoculation of mice with a sublethal dose of L. monocytogenes results in systemic infection involving the spleen and liver and induces innate and adaptive immune responses that clear the infection. More than 60% of Listeria is taken up by the liver within 10 min of i.v. inoculation (19, 20). Ab-mediated blockade of CD11b dramatically reduces the recruitment of myeloid cells to the liver and results in markedly increased growth of L. monocytogenes (21). Administration of the RB6-8C5 Ab, which binds to an epitope shared by Ly6C and Ly6G, depletes granulocytes and Ly6C<sup>hi</sup> monocytes and results in uncontrolled hepatic growth of L. monocytogenes (22). The relative contribution of granulocytes and Ly6C<sup>hi</sup> monocytes to hepatic clearance of L. monocytogenes remains unresolved.

The innate immune response during L. monocytogenes infection depends on rapid monocyte recruitment, a process that has been studied most extensively in the spleen (23, 24). Ly6C<sup>hi</sup> monocytes are recruited from bone marrow to spleen during L. monocytogenes infection, and they differentiate into TNF- and inducible NO synthase (iNOS)-producing DCs (TipDCs) that enhance bacterial clearance (23). The recruitment of Ly6C<sup>hi</sup> monocytes requires CCR2, a chemokine receptor that responds to MCP-1 and -3. CCR2<sup>-/-</sup> mice have decreased numbers of Ly6C<sup>hi</sup> monocytes in the periphery because monocytes are retained in the bone marrow, markedly increasing susceptibility to L. monocytogenes infection (24, 25). CCR2 deficiency also increases the severity of L. monocytogenes infection in the liver (8), but it remains unclear whether this results from defective recruitment of monocytes or other CCR2-expressing cell populations.

Although the role of CCR2 in monocyte egress from the bone marrow is established (24–29), it remains unresolved whether CCR2 also plays a role in monocyte trafficking into and within peripheral tissues. Some studies indicated that CCR2-mediated
signaling is required for monocyte entry into the CNS (7), atherosclerotic plaques (6), ischemic myocardium (30), and the peritoneal cavity (26) in the setting of tissue inflammation. However, it was also shown that the migration of monocytes from the bloodstream into the white pulp of L. monocytogenes-infected spleen is CCR2 independent (24). In the current study, we demonstrated that neither the targeting of Ly6C<sup>high</sup> monocytes from the bloodstream to hepatic foci of infection nor their bactericidal function in the liver requires CCR2-mediated signals. Our study demonstrated that trafficking of Ly6C<sup>high</sup> monocytes within the liver is not mediated by pertussis toxin-sensitive chemokine receptors but depends on integrin- and extracellular matrix-mediated adhesion.

Materials and Methods

**Mice and infections**

All mice used in this study were bred at Memorial Sloan-Kettering Research Animal Resources Center. The CCR2 reporter transgenic strain was previously generated in our laboratory using bacterial artificial chromosome carrying the gene encoding enhanced GFP (EGFP) under the control of CCR2 promoter (31). The CCR2<sup>−/−</sup>Tg(CCR2-EGFP) strain was generated by crossing the CCR2-reporter transgenic strain to a CCR2<sup>−/−</sup> background. Bone marrow chimeras were generated according to the standard protocol. Briefly, C57BL/6 mice were subjected to irradiation at a dose of 1000 Gy, followed by i.v. injection of 1 × 10<sup>6</sup> bone marrow cells bearing differential CD45.1/2 congenic markers. Mice were infected i.v. with 3 × 10<sup>6</sup> CFU<sub>B. pertussis</sub> strain 1040S, as described previously (32). At indicated times following infection, livers were harvested and dissociated in PBS containing 0.05% Triton X-100, and bacterial CFU numbers of Ly6C<sup>high</sup> monocytes were detectable within 1 d of infection. Bone marrow chimeras were generated according to the standard protocol. Briefly, C57BL/6 mice were subjected to irradiation at a dose of 1000 Gy, followed by i.v. injection of 1 × 10<sup>6</sup> bone marrow cells bearing differential CD45.1/2 congenic markers. Mice were infected i.v. with 3 × 10<sup>6</sup> CFU<sub>L. monocytogenes</sub> strain 1040S, as described previously (32). At indicated times following infection, livers were harvested and dissociated in PBS containing 0.05% Triton X-100, and bacterial CFU were determined by plating on brain-heart infusion agar plates.

**Flow cytometry**

At various times postinfection, livers were perfused, excised, and digested in collagenase/DNase, as previously described (33). Nonparenchymal cells were further purified using 40%/60% Percoll gradient. Cells at the interface of the upper and lower layer of Percoll solution were collected and subjected to Ab staining. The following Abs were purchased from BD Pharmingen (San Diego, CA): anti–CD11b-PerCP-Cy5.5 (M1/70), anti–Ly6C-FTTC (AL-21), anti–Ly6C-PE-Ab (2C3), streptavidin–allophycocyanin-Cy7, anti–Ly6C-FITC (5C7), and anti–CD45-IgG1 Alexa Fluor 647 (A20), anti–TNF–allophycocyanin–PerCP-Cy5.5 (MP6-XT22), and anti–CD11c–allophycocyanin–FL1.3 (HL.3). Anti–MHC class II–PE (M5/114.15.2) was purchased from eBioscience (San Diego, CA). Anti–CD45-Pacific Blue (30-F11) was purchased from Biolegend (San Diego, CA). Rabbit anti–iNOS Ab was purchased from Millipore (Billerica, MA). Alexa633-F(ab′)<sub>2</sub> of anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA). For intracellular TNF staining, liver non-parenchymal cells were incubated in RPMI 1640 medium in the presence of brefeldin A for 4 h without stimulation and processed according to the manufacturer’s protocol for Cytotox/Cytoperm (BD Pharmingen).

**Histology and immunofluorescence**

The left liver lobe was collected and fixed in 4% paraformaldehyde; 10-μm cryosections were prepared using Leica CM1900 UVICUT microtome (Leica Microsystems, Wetzlar, Germany). Infection foci were visualized by staining with L. monocytogenes antiserum (BD Biosciences, San Jose, CA), followed by staining with Alexa633-F(ab′)<sub>2</sub>-anti-rabbit IgG (Invitrogen). iNOS primary staining was performed using anti-iNOS Ab purchased from Millipore. ICAM-1 was stained by anti–ICAM-1–biotin (3E2) Ab purchased from BD Biosciences. CD31 was stained by anti–CD31 (MEC 13.3) Ab purchased from BD Biosciences. Nuclei were visualized with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired with Leica TCS SP2 AOBS laser scanning confocal microscope (Leica Microsystems) with a 20× 0.7 NA objective lens. Acquired images were processed and analyzed with Volocity software (PerkinElmer Impruvision, Waltham, MA).

**Cell sorting and transfer**

Bone marrow cells were collected from femurs and tibias. EGFP<sup>+</sup> cells were sorted by Memorial Sloan-Kettering Cancer Center’s flow cytometry core facility using a BD ARIA flow cytometer (BD Biosciences). NK1.1<sup>+</sup> cells were depleted using NK1.1-biotin (clone PK136 from eBioscience), following the MACS sorting protocol (Miltenyi Biotec, Auburn, CA). For labeling, cells were incubated with 2 μM CFSE in PBS or 4 μM 5-(and-6)-((4-chloromethyl)benzoyl)aminotetramethylrhodamine (CMTMR; both from Invitrogen) in RPMI 1640 for 15 min at 37˚C and washed twice in PBS. CMTMR-labeled cells were incubated in RPMI 1640 for an additional 20 min at 37˚C. Cells were counted and mixed to give a 1:1 ratio of CFSE- or CMTMR-labeled cells. The cell suspension was transferred into a recipient mouse by i.v. injection. For pertussis toxin treatment, dye-labeled cells were incubated with 200 ng/ml Bordetella pertussis toxin (Calbiochem, San Diego, CA) in RPMI 1640 with 2% FBS at 37˚C for 20 min and washed three times with PBS before transfer.

**Calcium influx assay**

A calcium influx assay was performed, as previously described (34), to confirm the inhibition of chemokine signaling by pertussis toxin treatment. Cells were labeled with indo-1 (Invitrogen), according to the product manual, and stimulated at 37˚C with 10 ng/ml MCP-1 that was prepared in our laboratory. Calcium influx was detected with a BD LSRII flow cytometer (BD Biosciences). One micromolar ionomycin was used as a positive control to induce calcium influx.

**In vivo Ab blocking**

F(ab′)<sub>2</sub> fragment of anti–CD11b, anti–CD44 Abs were prepared according to the manufacturer’s protocol for the F(ab′)<sub>2</sub> Preparation Kit (Pierce, Rockford, IL). A dose (4 mg/kg body weight) of CD11b (5C6), CD44 (IM7), ICAM-1 (YN1/1.7.4), CD11a (M17/4) blocking Ab or IgG control was injected i.v. into infected C57BL/6 mice at 12 h postinfection. Liver monocytes were analyzed by FACS at 6 h after treatment.

**Intravital microscopy**

Liver intravital microscopy was conducted, as previously described (35), on a Zeiss LSM 710 single-photon microscope (Oberkochen, Germany) with a 25× 1.0 NA lens. In brief, animals were anesthetized; an incision was made along the peritoneum to expose a single lobe of the liver; and the mouse was placed on an inverted microscope platform. Animals were provided with additional anesthesia, as needed, and oxygen during imaging. Blood flow was visualized by injecting 10-kDa fluorescent dextran during the imaging. L. monocytogenes was visualized by using a 1040S3 strain expressing red fluorescent protein. Image analysis was conducted with Volocity Software (PerkinElmer Impruvision).

**Statistics**

Statistical validation of data was done with the Student t test when the sample size was greater than five per group; the nonparametric Mann-Whitney U test was used when the sample size was three to five per group. All data are presented as the arithmetic mean ± SEM.

**Results**

Ly6C<sup>high</sup> monocytes are recruited to foci of L. monocytogenes infection in the liver

Hepatocytes constitute the major cell type in the liver parenchyma. Resident immune cells, including Kupffer cells, DCs, NK cells, NK T cells, and T cells, reside in the lumen of portal tracts and sinusoids (36). During steady state, Ly6C<sup>high</sup> monocytes represent ~1–3% of total CD45<sup>+</sup> cells in the liver. Systemic L. monocytogenes infection induces the influx of leukocytes into the liver, and increased numbers of Ly6C<sup>high</sup> monocytes are detectable within 1 d of infection (Fig. 1A). The number of Ly6C<sup>high</sup> monocytes in the liver increases as the infection progresses and peaks 3 to 4 d after bacterial inoculation, accounting for ~30% of CD45<sup>+</sup> cells in the liver (Fig. 1B). Upon bacterial clearance 10 d following infection, the number of Ly6C<sup>high</sup> monocytes in the liver decreases, eventually returning to steady-state levels 2 wk postinfection (Fig. 1B, 1C).

Using a CCR2-reporter mouse strain (31, 37), in which 95% of EGFP-expressing cells are Ly6C<sup>high</sup> monocytes and ~99% of Ly6C<sup>high</sup> monocytes express EGFP (Supplemental Fig. 1), we tracked Ly6C<sup>high</sup> monocytes. EGFP<sup>+</sup> cells that are not Ly6C<sup>high</sup> monocytes express NK1.1 and account for ~5% of total EGFP<sup>+</sup> cells. In uninfected mice, a small number of EGFP<sup>+</sup> cells are seen within the lumen of presinusoid venules, and very few are found within sinusoids. During L. monocytogenes infection, EGFP<sup>+</sup> cells surround foci of infection and form lesions within the liver (Fig. 1D).
However, the majority of EGFP⁺ monocytes are not infected, as determined by quantitative culture of FACS-sorted EGFP⁺ monocytes from infected livers. One million sorted monocytes yielded only 100–200 CFU L. monocytogenes, indicating that only 1 in 5,000–10,000 monocytes harbors living bacteria.

Ly6C<sup>high</sup> monocytes differentiate into TipDCs at foci of infection

Our previous studies showed that Ly6C<sup>high</sup> monocytes differentiate into TipDCs in the spleen and contribute to immune defense (9). To determine whether recruited monocytes undergo similar differentiation in the liver, we isolated leukocytes from infected liver and analyzed the expression of characteristic markers of TipDCs. Intracellular cytokine staining revealed high levels of TNF expression in CD11b<sup>high</sup>Ly6C<sup>high</sup> monocytes (Fig. 2A). Increased levels of iNOS expression were also detected in these monocytes by intracellular staining (Fig. 2B), and histologic analysis demonstrated that monocytes located within foci of infection express iNOS, suggesting that in vivo proximity to bacteria induces iNOS expression (Fig. 2D). These CD11b<sup>high</sup>Ly6C<sup>high</sup> monocytes express CD11c and MHC class II, albeit at various levels (Fig. 2C).

CCR2 deficiency results in impaired monocyte recruitment to the liver and greater bacterial burdens

Recruitment of Ly6C<sup>high</sup> monocytes to the liver was greatly diminished in CCR2-deficient mice during the steady state and after Listeria infection (Fig. 3A). Impaired monocyte recruitment resulted in a greater bacterial burden in the liver as early as 1 d following infection. Three days following inoculation with a normally sublethal dose of bacteria, CCR2<sup>−/−</sup> mice had 100-fold more L. monocytogenes in the liver than did wild type mice (Fig. 3B) and died of infection by day 5. Recruitment of CD11b<sup>high</sup>Ly6C<sup>int</sup>Ly6G<sup>+</sup> neutrophils was not diminished by CCR2 deficiency (Supplemental Fig. 2). The absolute number of neutrophils in the liver of infected CCR2-deficient mice was twice that of their wild type counterparts, most likely in response to increased hepatic infection in CCR2-deficient mice. Of note, splenic infection in CCR2<sup>−/−</sup> and wild type mice did not differ until day 3 of infection (23), whereas we detected a 5-fold difference in the liver 1 d after infection. This may result from a relative paucity of resident Ly6C<sup>high</sup> monocytes in liver compared with spleen prior to and during the early stages of infection.

Although previous studies demonstrated that Ly6C<sup>high</sup> monocytes accumulate in the bone marrow of CCR2<sup>−/−</sup> mice during L. monocytogenes infection, it remains unclear whether cell-autonomous expression of CCR2 is essential for egress from the bone marrow or whether emigration of a few CCR2-expressing monocytes is sufficient to promote the emigration of other monocytes. To address this issue, we generated bone marrow chimerial mice using irradiated wild type C57BL/6 mice as recipients of CCR2<sup>+/+</sup> and CCR2<sup>−/−</sup> donor bone marrow mixed in different ratios. Infection of these mice with L. monocytogenes confirmed a cell-intrinsic and essential role for CCR2 in the egress of Ly6C<sup>high</sup> monocytes from bone marrow (Fig. 3C). By changing the ratio of donor CCR2<sup>+/+</sup> to CCR2<sup>−/−</sup> bone marrow cells, we also demonstrated a correlation between bacterial clearance from the liver and the number of recruited CCR2<sup>+/+</sup> Ly6C<sup>high</sup> monocytes (Fig. 3D).

CCR2 is dispensable for Ly6C<sup>high</sup> monocyte trafficking from the bloodstream to hepatic foci of infection

Ly6C<sup>high</sup> monocytes circulating in the bloodstream express CCR2 and, thus, are presumed to be responsive to CCR2 ligands expressed...
by peripheral tissues. The expression of MCP-1 and -3, two chemokine ligands of CCR2, is readily detected in peripheral tissues, including spleen and liver (25). However, it remains unclear whether CCR2-mediated signals are required for monocyte trafficking into and within \textit{L. monocytogenes}-infected organs. To directly compare recruitment of CCR2$^{+/+}$ and CCR2$^{-/-}$ Ly6C$^{high}$ monocytes from the circulation to the liver, we injected mixtures of wild type and CCR2$^{-/-}$ monocytes into the bloodstream of

![FIGURE 3](image)

**FIGURE 3.** CCR2 deficiency results in impaired monocyte recruitment to the liver and higher bacterial burdens. \textit{A}, Representative FACS plots of liver cells gated on CD45$^+$ nonparenchymal cells and stained for CD11b and Ly6C. Cells were collected from C57BL/6 mice 3 d following \textit{L. monocytogenes} infection and incubated with brefeldin A for 4 h. \textit{A}, CD11b expression and TNF production by cells obtained from uninfected and infected mice were detected by flow cytometry. \textit{B}, iNOS expression by Ly6C$^{high}$ monocytes was determined by intracellular staining in uninfected and \textit{L. monocytogenes}-infected mice. \textit{C}, CD11b$^{high}$Ly6C$^{high}$ monocytes express CD11c and MHC class II. Dot plot is gated on CD45$^+$ cells (gray) and CD11b$^{high}$Ly6C$^{high}$ cells (green), showing the increased expression of CD11c and MHC class II by monocytes relative to that of other cell populations. \textit{D}, Immunofluorescence staining for iNOS was performed on liver sections of infected CCR2-reporter mice. DAPI staining identifies hepatocyte nuclei, and EGFP expression identifies Ly6C$^{high}$ monocytes (original magnification ×20; scale bar, 70 μm).
L. monocytogenes-infected recipient mice and monitored their targeting to the liver. First, CCR2-reporter mice were crossed to the CCR2\textsuperscript{\textasciitilde2} background to obtain the CCR2\textsuperscript{\textasciitilde2}/Tg(CCR2-EGFP) strain. We sorted EGFP\textsuperscript{+} bone marrow cells, in which NK1.1\textsuperscript{+} cells had been depleted, from CCR2\textsuperscript{+/+} Tg(CCR2-EGFP) and CCR2\textsuperscript{\textasciitilde2}/Tg(CCR2-EGFP) mice that had been infected 6 h previously with L. monocytogenes, differentially labeled them with fluorescent dyes in vitro, and then mixed cells at a 1:1 ratio prior to transfer into infected C57BL/6J recipient mice (Fig. 4A). We retrieved comparable numbers of transferred CCR2\textsuperscript{\textasciitilde2} or CCR2\textsuperscript{\textasciitilde2} Ly6C\textsuperscript{high} monocytes from the liver 12 h after cell transfer (Fig. 4B). Histologic analysis indicated CCR2\textsuperscript{\textasciitilde2} and CCR2\textsuperscript{\textasciitilde2} monocytes localized equivalently to foci of infection (Fig. 4C).

We monitored trafficking of monocytes in the livers of infected CCR2\textsuperscript{\textasciitilde2} and CCR2\textsuperscript{\textasciitilde2} CCR2-reporter mice by intravital microscopy. EGFP\textsuperscript{+} cells accumulated at foci of infection in both mice, although the density of EGFP\textsuperscript{+} cells was greater in CCR2\textsuperscript{+/+} mice, reflecting markedly decreased emigration of EGFP\textsuperscript{+} cells from the bone marrow of CCR2\textsuperscript{\textasciitilde2} mice. The movement of EGFP\textsuperscript{+} cells in areas proximal to foci of infection was similar in CCR2\textsuperscript{+/+} and CCR2\textsuperscript{\textasciitilde2} mice, suggesting that CCR2 is not required for movement within the liver (Supplemental Videos 1, 2). Of note, monocytes that entered the center of foci of infection became less mobile, potentially as a result of decreased blood perfusion to these regions. To further characterize the relative trafficking of CCR2\textsuperscript{\textasciitilde2} and CCR2\textsuperscript{\textasciitilde2} monocytes in the L. monocytogenes-infected liver, we adoptively transferred bone marrow monocytes from CCR2\textsuperscript{+/+} or CCR2\textsuperscript{\textasciitilde2} donor mice, each expressing EGFP under the CCR2 promoter, into the bloodstream of infected wild type recipient mice and recorded their trafficking in the regions adjacent to infected hepatic foci (Supplemental Videos 3, 4). Quantitative analysis of EGFP\textsuperscript{+} donor-cell trafficking showed no difference in the speed, arrest, or linearity (meandering index) between monocytes sufficient or deficient for CCR2 (\(p = 0.57\), \(p = 0.37\), and \(p = 0.84\), respectively) (Fig. 4D).

These results indicated that CCR2-mediated signals do not contribute to trafficking of monocytes within the L. monocytogenes-infected liver.

**FIGURE 4.** CCR2 is dispensable for the recruitment of Ly6C\textsuperscript{\textasciitilde2} monocytes from the circulation to foci of infection in the liver. A, Ly6C\textsuperscript{\textasciitilde2} monocytes were sorted from the bone marrow of infected CCR2\textsuperscript{\textasciitilde2} Tg(CCR2-EGFP) and CCR2\textsuperscript{\textasciitilde2} Tg(CCR2-EGFP) mice based on their expression of EGFP, and NK1.1\textsuperscript{+} cells were depleted by MACS sorting. Monocytes were differentially labeled with CFSE or CMTMR fluorescent dyes in vitro, mixed at a 1:1 ratio, and transferred into infected C57BL/6J recipient mice. Livers of recipients were harvested 12 h later. B, Representative FACS plot gated on liver Ly6C\textsuperscript{\textasciitilde2} monocytes, showing the percentage of transferred CCR2\textsuperscript{\textasciitilde2} and CCR2\textsuperscript{\textasciitilde2} cells. C, Immunofluorescence analysis of liver sections of recipients. CCR2\textsuperscript{\textasciitilde2} monocytes were labeled with CFSE (green), whereas CCR2\textsuperscript{\textasciitilde2} monocytes were labeled with CMTMR (red) (original magnification \times20; scale bar, 70 \&mu;m). D, EGFP\textsuperscript{+} monocytes sorted from the bone marrow of infected CCR2\textsuperscript{\textasciitilde2} Tg(CCR2-EGFP) or CCR2\textsuperscript{\textasciitilde2} Tg(CCR2-EGFP) mice were injected into infected C57BL/6J recipient mice. Liver intravital microscopy was conducted following cell transfer. Individual cells were plotted based on trafficking-behavior indices from image analysis.
CCR2 is dispensable for the bactericidal function of Ly6C<sup>high</sup> monocytes in the liver

To test whether monocytes lacking CCR2 control infection in the liver, we transferred sorted EGFP<sup>+</sup> bone marrow cells from CCR2<sup>-/-</sup> or CCR2<sup>+/+</sup> Tg(CCR2-EGFP) donors into infected CCR2<sup>-/-</sup> recipients and measured the intensity of infection 24 h later. We observed significant and similar reduction of liver bacterial counts in recipients receiving CCR2<sup>+/+</sup> or CCR2<sup>-/-</sup> monocytes compared with mice receiving no transferred monocytes (Fig. 5). This indicated that monocytes that emigrated from the bone marrow and entered the bloodstream traffic to hepatic sites of infection provide protection against <i>L. monocytogenes</i> in a CCR2-independent manner.

Chemokine-independent adhesion mechanisms mediate the targeting of monocytes to foci of infection

Because CCR2 is not required for the targeting of Ly6C<sup>high</sup> monocytes to foci of hepatic infection, we asked whether chemokine receptor-mediated signals in general are required. To address this, we treated EGFP<sup>+</sup> bone marrow cells from CCR2-reporter mice in vitro with pertussis toxin, which blocks G protein-coupled receptors, prior to transfer into a <i>L. monocytogenes</i>-infected recipient mouse (Fig. 6A, 6B). Retention and targeting of pertussis toxin-treated and untreated monocytes to the liver and to foci of infection were comparable, as determined by flow cytometry and histologic analysis (Fig. 6C).

Chemokine receptor-independent targeting of monocytes from the bloodstream to the liver suggested that monocytes might be targeted to foci of infection by other mechanisms. Stimulated monocytes express high levels of surface Mac-1 integrin (38), which consists of CD11b and CD18 subunits and can serve as a receptor for ICAM-1 (39). To determine whether <i>L. monocytogenes</i> infection induces ICAM-1 expression in the liver and whether induction in the liver is localized to foci of infection, we

**FIGURE 6.** Chemokine-independent targeting of monocytes to the foci of infection. A. Ly6C<sup>high</sup> monocytes were sorted from the bone marrow of infected CCR2<sup>+/+</sup> Tg(CCR2-EGFP) mice. Monocytes were treated with 200 ng/ml pertussis toxin or PBS and differentially labeled with CFSE or CMTMR fluorescent dyes in vitro. A mixture of toxin- and control-treated monocytes (1:1 ratio) was transferred into infected C57BL/6J recipient mice. Livers of recipients were harvested 12 h after cell transfer. B. MCP-1–induced calcium influx was inhibited by pertussis toxin treatment. Control of treated monocytes showing the percentage of transferred pertussis toxin-treated and control cells. Immunofluorescence analysis of liver sections of recipients is shown. Pertussis toxin-treated monocytes were labeled with CFSE (green), control monocytes were labeled with CMTMR (red), and hepatocyte nuclei are stained with DAPI (original magnification ×20; scale bar, 70μm.).
performed immunofluorescence staining of infected livers and observed induction of ICAM-1 in regions immediately adjacent to foci of L. monocytogenes infection (Fig. 7A). ICAM-1 expression partially colocalizes with the endothelial marker CD31 (Fig. 7B), suggesting that sinusoidal endothelial cells upregulate ICAM-1 expression in response to bacterial molecules. Ab-mediated blockade of CD11b or ICAM-1 reduced retention of Ly6C<sup>high</sup> monocytes in liver sinusoids and their accumulation at infected foci. In contrast, Ab-mediated blockade of CD11a, a subunit of LFA-1 that also binds ICAM-1, did not reduce monocyte recruitment to the liver (Fig. 7C, 7D). Focal staining for ICAM-1 persisted when monocyte recruitment was inhibited by CD11b blockade or in CCR2-deficient mice (Fig. 7C), indicating that induced ICAM-1 is expressed by nonmonocytes. Nevertheless, because monocytes also express ICAM-1, it is possible that recruited monocytes enhance trafficking of circulating monocytes to foci of infection in a positive feedback loop. CD44 was also demonstrated to function as a surface molecule that sequesters neutrophils in inflamed liver sinusoids (40). By blocking CD44, we observed 50–60% reduction of monocyte recruitment to the liver (Fig. 7D). Ab-mediated blockade of CD11b, ICAM-1, or CD44 did not result in the accumulation of monocytes in the bone marrow, indicating the trafficking was not blocked at the interface between bone marrow and circulation. Thus, our results suggest that monocyte recruitment within the liver is mediated by chemokine-independent adhesion mechanisms involving CD11b, CD44, and ICAM-1.

Discussion

Recruitment and targeting of inflammatory cells during infection is a complex process involving multiple steps that are enabled, in part, by chemokine-dependent signals. In this study, we demonstrated that Ly6C<sup>high</sup> monocytes are rapidly recruited from bone marrow to the liver during L. monocytogenes infection. CCR2 deficiency impairs recruitment and leads to a greater bacterial burden in the liver. We provided evidence that CCR2-mediated signals principally enable monocyte egress from bone marrow and that once Ly6C<sup>high</sup> monocytes migrate into the circulation, they are targeted to hepatic foci of infection and provide immune defense, without a requirement for additional CCR2-mediated signals. Our study indicated that monocyte recruitment to hepatic foci involves the focal induction of adhesion molecules.

Our histologic data and previous studies (8) showed that the distribution of bacterial infection in the liver is localized and restricted to inflammatory foci that consist of recruited leukocytes and infected hepatocytes. Intravital imaging demonstrated that blood flow to the central region of infected hepatic foci was markedly diminished. Bacterial infection seems to be restricted by the recruitment of inflammatory monocytes to the region surrounding bacterially infected foci. In contrast to previous studies, in which leukocyte recruitment to the liver was studied in the setting of widespread inflammation (29, 40), our study focused on monocyte recruitment to focal sites of infection and inflammation. We found that bacterial infection selectively increased expression of the adhesion molecule ICAM-1 in regions immediately adjacent to infected foci. Focal induction of ICAM-1 during L. monocytogenes infection may result from localized production and diffusion of TLR ligands from areas of bacterial infection and hepaticocyte necrosis.

Leukocyte recruitment in liver sinusoids occurs by distinct mechanisms that differ from the tether-and-roll paradigm governing the adhesion and emigration in other vascular compartments (41). Hepatic sinusoidal endothelium does not express selectins, and selectins are not essential for recruitment of cells to sinusoids (42). In fact, visualization of leukocyte movement in the liver revealed that adhesion in sinusoids often occurred independent of rolling (41). Hepatic sinusoids are fenestrated and lack a basal lamina and tight junctions. Thus, the recruitment of monocytes from sinusoids to foci of infection likely follows a scheme that differs from chemokine-dependent endothelial transmigration. Although most previous studies showed that leukocyte chemotaxis induced by chemokines is inhibited by pertussis toxin, certain chemokine receptors were shown to couple to pertussis toxin-sensitive and -resistant G proteins (43). Therefore, it is possible that pertussis toxin-insensitive signals mediated by chemokine receptors contribute to monocyte recruitment.
Previous studies showed that neutrophil sequestration in inflamed liver sinusoids following systemic LPS administration is mediated by the interaction of CD44 on neutrophils with extracellular hyaluronan (HA) (40). Systemic administration of LPS, in addition to resulting in hepatic inflammation, induces the expression of serum-derived HA-associated protein, which enhances the association of HA with CD44. In our experiments, we found that Ab-mediated blockade of CD44 markedly reduced the number of Ly6Chigh monocytes detected by FACS in L. monocytogenes-infected livers (Fig. 7D). Thus, our studies demonstrated a role for CD44 in inflammatory monocyte recruitment to the liver. However, it is unclear whether L. monocytogenes infection induces serum-derived HA-associated protein expression and, thus, promotes monocyte recruitment by enhancing interactions between CD44 and HA or whether HA expression is altered in areas adjacent to foci of L. monocytogenes infection. Further studies are necessary to determine how CD44 contributes to the targeting of monocytes to liver during L. monocytogenes infection.

Studies with CX3CR1-GFP mice using intravital microscopy documented the patrolling behavior of resident monocytes in noninflamed dermis (44). In those studies, specific Ab-mediated blockade of LFA-1 led to detachment of CX3CR1-expressing noninflamed dermis (44). In our experiments, specific Ab-mediated blockade of CD44 markedly reduced the number of Ly6Chigh monocytes detected by FACS in L. monocytogenes-infected livers (Fig. 7D). Thus, our studies demonstrated a role for CD44 in inflammatory monocyte recruitment to the liver. However, it is unclear whether L. monocytogenes infection induces serum-derived HA-associated protein expression and, thus, promotes monocyte recruitment by enhancing interactions between CD44 and HA or whether HA expression is altered in areas adjacent to foci of L. monocytogenes infection. Further studies are necessary to determine how CD44 contributes to the targeting of monocytes to liver during L. monocytogenes infection.

In summary, we demonstrated that Ly6Chigh monocytes are rapidly recruited from bone marrow to foci of infection in the liver where they mediate clearance of L. monocytogenes. Although monocyte egress from bone marrow is CCR2 dependent, entry into the liver parenchyma and localization to foci of bacterial infection are chemokine receptor independent. Focal induction of ICAM-1 facilitates the targeting of inflammatory monocytes to regions immediately adjacent to foci of bacterial infection. Ly6Chigh monocytes are increasingly recognized as essential mediators of defense against viral, bacterial, fungal, and protozoan pathogens (8, 10, 11, 13, 45–47). Deciphering the mechanisms that enable monocyte trafficking to peripheral sites of bacterial infection may provide new approaches to enhance immune defense against these pathogens.

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Disclosures

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References


Corrections


The authors have revised the funding information in the footnotes to include an additional grant. The corrected footnote is below.

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