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Ly6G+ Neutrophils Are Dispensable for Defense against Systemic Listeria monocytogenes Infection

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Listeria monocytogenes is a facultative intracellular bacterium that causes systemic infections in immunocompromised hosts. Early recruitment of myeloid cells, including inflammatory monocytes and neutrophils, to sites of L. monocytogenes infection is essential for the control of infection and host survival. Because previous experimental studies used depleting or blocking Abs that affected both inflammatory monocytes and neutrophils, the relative contributions of these cell populations to defense against L. monocytogenes infection remain incompletely defined. In this article, we used highly selective depletion strategies to either deplete inflammatory monocytes or neutrophils from L. monocytogenes-infected mice and demonstrate that neutrophils are dispensable for early and late control of infection. In contrast, inflammatory monocytes are essential for bacterial clearance during the innate and adaptive phases of the immune response to L. monocytogenes infection.

Neutrophils and monocytes are distinct myeloid cell populations that contribute to innate immune defense against microbial pathogens (1, 2). Neutrophils constitute 50–60% of circulating WBCs and are ~10-fold more numerous than circulating monocytes. Granulocytes are considered terminally differentiated cells that express a range of antimicrobial effector functions. In contrast, circulating monocytes are pluripotent and, upon recruitment to sites of infection, can respond to the inflammatory context by differentiating into distinct effector populations expressing antimicrobial factors. The risk of microbial infection is markedly increased when mice or humans develop profound neutropenia (3). Upon recruitment to foci of infection, neutrophils phagocytose and kill microorganisms by generating reactive oxygen species and releasing granules that contain antimicrobial factors (4, 5). Neutrophil-mediated release of granules and chromatin generates neutrophil extracellular traps that sequester and kill extracellular microbes (6–8). Neutrophils also secrete chemokines and recruit additional immune cells (9). In mice, circulating neutrophils specifically express the Ly6G cell surface protein (10).

Circulating monocytes enter the bloodstream from the bone marrow (11) and also contribute to antimicrobial defense. Monocytes are divided into subsets on the basis of chemokine receptor expression and the presence of specific surface markers (12).

In mice, expression of high levels of Ly6C and CCR2 identifies a monocyte subset termed inflammatory or Ly6Cinh monocytes. This population of monocytes represents ~2–3% of circulating WBCs in an uninfected mouse and is rapidly recruited to sites of infection and inflammation. Inflammatory monocytes play essential roles in immune defense against various pathogens (2). Neutrophils and inflammatory monocytes share some antimicrobial effector functions, such as expression of phagocyte oxidase and secretion of TNF, and they are often recruited in tandem to sites of infection. However, the mechanisms and triggers inducing their recruitment and activation are distinct, and their contributions to antimicrobial clearance are generally nonoverlapping.

Listeria monocytogenes, a Gram-positive facultative intracellular bacterium, causes severe disease in immunocompromised hosts and is acquired by ingestion of contaminated food (13, 14). Intravenous inoculation of mice with L. monocytogenes results in systemic infection and recruitment of neutrophils and monocytes to the spleen and liver. Early recruitment of inflammatory cells, mediated by CD11b, is essential for the control of L. monocytogenes infection (15) and sets the stage for adaptive immune responses, which ultimately clear the infection (16). Depletion of inflammatory cells by administering the depleting RB6-8C5 mAb specific for granulocyte receptor 1 (Gr-1) markedly enhanced L. monocytogenes infection and suggested that neutrophils play a major role in defense (17–21). At the time of these experiments, however, it was not appreciated that the RB6-8C5 mAb associates with an epitope shared by the Ly6C and Ly6G proteins (22) and, thus, binds to and depletes neutrophils and inflammatory monocytes (2). Although a specific role for inflammatory monocytes in defense against L. monocytogenes has been revealed by studies in mice deficient for CCR2 (23, 24), a chemokine receptor that is required for monocyte recruitment (25), the relative contribution of neutrophils to defense against L. monocytogenes infection remains unresolved.

In this study, we specifically and selectively depleted inflammatory monocytes or neutrophils from mice during infection with L. monocytogenes. Inflammatory monocytes were depleted using a recently described CCR2-DTR mouse strain, and neutrophils

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Abbreviations used in this article: DC, dendritic cell; DT, diphtheria toxin; Gr-1, granulocyte Receptor 1; Tg, transgenic.

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were depleted using the Ly6G-specific mAb 1A8 (10, 22), which deplete neutrophils but not Ly6C<sup>hi</sup> monocytes (26). We demonstrate that inflammatory monocytes but not neutrophils are essential in control L. monocytogenes infection.

Materials and Methods

Mice and infections

All mice used in this study were bred at Memorial Sloan-Kettering Research Animal Resources Center. C57BL/6 and OT-1 TCR transgenic (Tg) mice were purchased from The Jackson Laboratory. The CCR2-GFP and CCR2-DTR Tg strains were described previously (27, 28). Mice were infected i.v. with 3 × 10<sup>3</sup> or 5 × 10<sup>3</sup> L. monocytogenes strain 10403S. For primary or secondary challenges, respectively, or 1 × 10<sup>5</sup> LM-OVA, as described previously (29).

At the 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.

mAb and diphtheria toxin treatment

Gr-1–specific RB6-8C5 and Ly6G-specific 1A8 mAbs were purified from hybridoma supernatants. C57BL/6 mice were injected i.p. with either 250 µg/dose 1A8 or RB6-8C5 Abs following L. monocytogenes inoculation and at indicated time points.

Flow cytometry

At various times points, blood, spleens, and livers were collected and prepared as described previously (30). Nonparenchymal cells from livers were further purified using 40/80% Percoll gradients. Cells at the interface of the Percoll layers were collected and subjected to Ab staining (28).

The following fluorescent Abs were purchased from BD Pharmingen (San Diego, CA): anti-CD11b (Mil70), Ly6C (AL-21), Ly6G (1A8), CD4 (RM4-5), Thy1.1 (OX-7), Thy1.2 (30-H12), CD44 (IM7), IFN-γ (XMG1.2), and Foxp3 (FJK-16s). Anti-CD45 (30-F11), CD45.1 (A20), and CD62L (DREG-56) were purchased from BioLegend (San Diego, CA). Staining for intracellular IFN-γ, Foxp3 was performed using the Foxp3 staining kit from eBioscience (San Diego, CA) according to the product manual. FACs was performed with an LSR II, and data were analyzed with FlowJo software. For morphological examination, cell subsets were obtained by FACs sorting with the purity of >98%. Cells were spun onto glass slides and stained using Diff-Quik Satin Set (Dade Behring).

Adaptive transfers of OT1 cells and in vitro T cell restimulation

Naive CD8 OT1 TCR Tg cells were isolated from spleens of OT1 TCR Tg mice using a CD8<sup>+</sup> isolation kit from Miltenyi Biotec followed by further FACs sorting for CD4<sup>+</sup>CD62L<sup>hi</sup> population. A total of 2 × 10<sup>5</sup> purified OT1 cells were injected i.v. into naive recipients 1 d before infection. T cells were isolated from spleens at day 7.5 postinfection, restimulated in vitro with peptide-pulsed dendritic cell (DC) in the presence of brefeldin A for 5 h, and then subjected to intracellular cytokine staining.

Histology and immunofluorescence

The spleen or the left lobe of the liver were collected and fixed in 4% paraformaldehyde; 10-µm cryosections were prepared using a Leica CM1900UV microtome (Leica Microsystems, Wetzlar, Germany). Foci of infection were visualized by staining with L. monocytogenes antiserum (BD Biosciences, San Jose, CA), followed by staining with Alexa 633-F(ab’)<sub>2</sub>-anti-rabbit IgG (Invitrogen). Neutrophils were visualized by anti-Ly6G (1A8) staining followed by staining with Alexa 594-anti-rat IgG. Nuclei were visualized with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired with Leica TCS SP2 AOB laser scanning confocal microscope (Leica Microsystems) with a ×20 0.7 NA objective lens.

Acquired images were processed and analyzed with Velocity software (PerkinElmer Imropvisation, Waltham, MA).

Statistics

The unpaired Student t test and log-rank (Mantel–Cox) test were used for statistical analyses with GraphPad Prism software. The p values were calculated by t test: *p < 0.05, **p < 0.01, and ***p < 0.001. A p value < 0.05 was considered statistically significant.
was considered statistically significant. All data are presented as the arithmetic mean ± SEM.

Results

Monocytes and neutrophils are recruited during L. monocytogenes infection

Systemic L. monocytogenes infection induces emigration of Ly6Ch* monocytes and neutrophils from the bone marrow. Increased numbers of circulating monocytes, which are identified as CD11bintLy6Ch*Ly6G−, and neutrophils, which are identified as CD11bhiLy6CintLy6G+, are detectable within 1 d of infection (Fig. 1A). As expected, monocytes and neutrophils differ in size and have distinct nuclear morphologies (Fig. 1B). Ly6Ch* monocytes and neutrophils were recruited to the spleen and liver postinfection of L. monocytogenes (Fig. 1C). To determine the localization of monocytes and neutrophils in the spleens and livers of infected mice, we used CCR2-GFP reporter mice to detect inflammatory monocytes and staining with a Ly6G-specific mAb to detect neutrophils (Fig. 1D). Whereas the majority of neutrophils localized to the red pulp of the spleen, Ly6Ch* monocytes aggregated within the white pulp in areas of L. monocytogenes infection. In the liver, monocytes and neutrophils were also recruited to foci of infection. Neutrophils localized to the center of bacterial foci of infection, whereas monocytes remained more peripheral, forming a ring around the site of infection (Fig. 1D) (28). The different localization of monocytes and neutrophils in hepatic foci of infection suggests that the two cell types make distinct contributions to innate defense against L. monocytogenes infection.

Differential depletion of monocytes and neutrophils

To characterize the roles of neutrophils and monocytes during L. monocytogenes infection, we developed an experimental strategy to selectively deplete these cell populations from infected mice. The anti–Gr-1 mAb RB6-8C5 has been used extensively to deplete neutrophils in mice and to investigate the role of these cells in host defense.
defense to various infections (31–35). Because RB6-8C5 depletes both neutrophils and inflammatory monocytes (10), it is impossible to attribute phenotypic changes to one or the other cell population. Although blood monocytes can be depleted by i.v. injection of clodronate liposomes (36), this approach also depletes tissue macrophages and often increases circulating neutrophils because of reagent induced inflammation (32). Recently, the specific depletion of inflammatory monocytes using a CCR2-DTR mouse strain has been described previously (27). Administration of DT to CCR2-DTR mice results in 99% depletion of inflammatory monocytes that persists for 3–4 d. Specific depletion of neutrophils is achieved by injecting a Ly6G-specific mAb 1A8 (37). No overt toxicity is observed in RB6-8C5, 1A8, or DT-treated uninfected mice, and, in the absence of infection, treated mice remained healthy for at least 14 d. To test the effectiveness of neutrophil and monocyte depletion strategies, 1A8, RB6-8C5, and DT were injected into C57BL/6 mice and CCR2-DTR mice, respectively, following L. monocytogenes inoculation and during active infection. Circulating monocytes and neutrophils in the blood were monitored daily to measure the extent of cellular depletion (Fig. 2A, 2B). The accumulation of monocytes and neutrophils to spleens and livers was abolished by respective depletions of these cell populations (Fig. 2C, 2D). Of note, the recruitment of monocytes is not diminished by anti-Ly6G treatment, suggesting neutrophils do not contribute to monocyte recruitment. Decreased numbers of neutrophils were recovered from the spleens of mice in which monocytes were depleted. This likely reflects increased cell death resulting from high bacterial burdens. Recruitment and positioning of monocytes or neutrophils at sites of infection in the spleen and liver are not affected by the depletion of either population (Fig. 3).

**Inflammatory monocytes but not neutrophils are essential for control of L. monocytogenes infection**

Although previous studies have suggested that neutrophils play an essential role in early immune defense against L. monocytogenes infection (17–21), we found that mAb 1A8-mediated depletion of Ly6G+ neutrophils did not adversely affect survival of infected mice (Fig. 4A). Furthermore, depletion of neutrophils 4–7 d post-infection also did not significantly impact survival (Fig. 4A). Cultures of spleens and livers from neutrophil-depleted mice at the end of the 16-d observation period revealed complete clearance of L. monocytogenes. In contrast, mice rapidly succumbed to L. monocytogenes infection when monocytes were depleted by DT administration to CCR2-DTR mice or anti-Gr-1 Ab administration to C57BL/6 mice in the early stage of infection (Fig. 4A), with markedly increased bacterial counts in spleens (Fig. 4B) and liver (Fig. 4D) at day 3 postinfection. Treatment of CCR2-DTR mice with DT on days 4 through 7 following L. monocytogenes inoculation also resulted in increased mortality (Fig. 4A) and significantly higher numbers of bacteria in the spleen (Fig. 4C) and liver (Fig. 4E), as determined on day 8 postinfection. These

![FIGURE 4.](http://www.jimmunol.org/) Inflammatory monocytes but not neutrophils are essential in control L. monocytogenes infection. C57BL/6 or CCR2-DTR mice were infected with 3000 L. monocytogenes. Anti-Ly6G 1A8 Ab or anti–Gr-1 RB6-8C5 Ab was injected i.p. upon infection and daily during the periods indicated. DT was injected upon infection and at every other day during the periods indicated. A, Survival of infected mice under three different depletion regimens. Ab or DT was administrated at periods of days 0–3 (blue), days 4–7 (red), and days 0–7 (green). Ten to 12 mice per group were used. The p values were derived from the comparisons of each treatment group to untreated group and are indicated in parentheses. B, Survival of infected mice under three different depletion regimens. Ab or DT was administrated at periods of days 0–3 (blue), days 4–7 (red), and days 0–7 (green). Ten to 12 mice per group were used. The p values were derived from the comparisons of each treatment group to untreated group and are indicated in parentheses. C, Survival of infected mice under three different depletion regimens. Ab or DT was administrated at periods of days 0–3 (blue), days 4–7 (red), and days 0–7 (green). Ten to 12 mice per group were used. The p values were derived from the comparisons of each treatment group to untreated group and are indicated in parentheses. D, Survival of infected mice under three different depletion regimens. Ab or DT was administrated at periods of days 0–3 (blue), days 4–7 (red), and days 0–7 (green). Ten to 12 mice per group were used. The p values were derived from the comparisons of each treatment group to untreated group and are indicated in parentheses. E, Survival of infected mice under three different depletion regimens. Ab or DT was administrated at periods of days 0–3 (blue), days 4–7 (red), and days 0–7 (green). Ten to 12 mice per group were used. The p values were derived from the comparisons of each treatment group to untreated group and are indicated in parentheses.
results indicate that Ly6C<sup>hi</sup> monocytes and monocyte-derived cells, but not neutrophils, are essential for effective immune defense against *L. monocytogenes* infection.

**T cell responses are not affected by depletion of neutrophils**

Early inflammatory responses influence T cell responses to *L. monocytogenes*. Although previous studies have implicated neutrophils in CD8<sup>T</sup> cell priming following *L. monocytogenes* infection (38), the in vivo depletion approaches that were used could not distinguish between neutrophils and monocytes. To determine whether depletion of Ly6C<sup>hi</sup> monocytes or neutrophils affects T cell responses, we depleted inflammatory monocytes or neutrophils 4–7 d following infection, and *L. monocytogenes*-specific CD4 and CD8 T cells responses were measured. CD4<sup>T</sup> T cell responses were measured by intracellular cytokine staining after stimulation of T cells from the spleen with DCs coated with the immunodominant LLO<sub>190–201</sub> epitope. CD8<sup>T</sup> T cell responses were measured by adoptively transferring OT-1 TCR transgenic cells into recipient mice followed by infection with a strain of *L. monocytogenes* that secretes OVA (LM-OVA). Treatment with anti-Ly6G Ab did not change the frequency of IFN-γ–producing, LLO<sub>190–201</sub>-specific CD4<sup>T</sup> T cells or IFN-γ production by adoptively transferred OT1 cells. In contrast, monocyte depletion markedly impaired IFN-γ expression by CD4<sup>T</sup> T cells, whereas the OVA-specific CD8<sup>T</sup> T cell response remained intact (Fig. 5A–C). Mice with continuous depletion of neutrophils from days 0 to 7 resolved the infection and were protected 21 d following primary infection from rechallenge with a lethal dose (Fig. 5D), indicating that T cell-mediated protective immunity against *L. monocytogenes* does not depend on neutrophils.

**Discussion**

Neutrophils efficiently and expeditiously clear extracellular pathogens by releasing bactericidal granules and producing neutrophil extracellular traps (5, 6). Patients with severe neutropenia are particularly vulnerable to infections caused by extracellular pathogens but not infection by intracellular bacteria. Thus, it has been difficult to reconcile the clinically and experimentally defined role for neutrophils in defense against extracellular bacterial infection with experimental studies suggesting a requirement for neutrophils in defense against *L. monocytogenes* infection. Recent studies have demonstrated that depletion of Gr-1<sup>+</sup> but not Ly6G<sup>+</sup> myeloid cell populations exacerbates infections caused by intracellular pathogens, including *Toxoplasma gondii* (37), mycobacteria (39), HSV type 1 (40), and certain strain of influenza (41). Our studies extend the spectrum of infections for which neutrophils are dispensable to include *L. monocytogenes* and also demonstrate, through the use of CCR2-depleter mice, that the susceptibility phenotype associated with Gr-1 depletion is attributable exclusively to inflammatory monocyte depletion. Our study, together with others using different intracellular microbial pathogens, suggest that microbial escape from the extracellular environment into intracellular compartments of host cells is an effective strategy to evade neutrophil-mediated killing.
Disclosures
The authors have no financial conflicts of interest.

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