

# CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> Immature Myeloid Cells Recruited in Response to *Salmonella enterica* Serovar Typhimurium Infection Exhibit Protective and Immunosuppressive Properties

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Immature myeloid cells in bone marrow are a heterogeneous population of cells that, under normal conditions, provide tissues with protective cell types such as granulocytes and macrophages. Under certain pathological conditions, myeloid cell homeostasis is altered and immature forms of these cells appear in tissues. Murine immature myeloid cells that express CD11b and Ly6C or Ly6G (two isoforms of Gr-1) have been associated with immunosuppression in cancer (in the form of myeloid-derived suppressor cells) and, more recently, infection. Here, we found that CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells accumulated and persisted in tissues of mice infected with *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). Recruitment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> but not CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells from bone marrow into infected tissues depended on chemokine receptor CCR2. The CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells exhibited a mononuclear morphology, whereas the CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells exhibited a polymorphonuclear or band-shaped nuclear morphology. The CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells differentiated into macrophage-like cells following *ex vivo* culture and could present antigen to T cells *in vitro*. However, significant proliferation of T cells was observed only when the ability of the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells to produce nitric oxide was blocked. CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells recruited in response to *S. Typhimurium* infection could also present antigen to T cells *in vivo*, but increasing their numbers by adoptive transfer did not cause a corresponding increase in T cell response. Thus, CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> immature myeloid cells recruited in response to *S. Typhimurium* infection exhibit protective and immunosuppressive properties that may influence the outcome of infection.

**S**almonellae are a leading and increasing cause of morbidity and mortality in humans worldwide (1, 2). Infections with salmonellae range in severity from self-limiting gastroenteritis to typhoid fever. Nontyphoidal salmonellae such as *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) are a significant cause of inflammatory enterocolitis and death due to food-borne illness and an emerging cause of invasive bacteremia in immunocompromised hosts. Typhoidal salmonellae such as *Salmonella enterica* serovar Typhi (*S. Typhi*) cause systemic infections characterized by bacterial penetration of the intestinal barrier and extraintestinal dissemination to the liver and spleen, where the microorganisms survive and replicate in professional phagocytes. Septic shock and death can occur if systemic infections with salmonellae are left untreated (1, 2).

Much of what is known about immunity to salmonellae comes from experimental infection of mice with *S. Typhimurium*, which has served as a useful model for the human disease caused by *S. Typhi* (3, 4). In humans and animal hosts, *S. Typhimurium* induces acute immunosuppression and delays the onset of protective immune responses (3–6). Immunity that eventually develops against *S. Typhimurium* requires humoral and cell-mediated immune responses (3). T cells, particularly gamma interferon (IFN- $\gamma$ )-producing CD4<sup>+</sup> T cells, play a critical role in the clearance of *S. Typhimurium* (3), but T cell responses to *S. Typhimurium* are thwarted during infection by mechanisms that, despite recent progress (7–12), are not well understood.

During early stages of infection, macrophages and neutrophilic granulocytes are critical for controlling spread and growth of *S. Typhimurium* (3). These cells are produced by the differentiation of immature myeloid cells in infected tissues and bone marrow (13–15). Although immature myeloid cells play a critical role in

the protective host response to infection (14), several early studies have linked nitric oxide (NO)-producing macrophage precursors to immunosuppression in salmonellosis (16). Murine immature myeloid cells express surface CD11b and Gr-1 (13) and have been found in spleens of mice infected with *S. Typhimurium* (17, 18). CD11b<sup>+</sup> Gr-1<sup>+</sup> immature myeloid cells are a heterogeneous population of cells that can be further divided based on the surface expression of Ly6C and Ly6G, two isoforms of Gr-1 that are differentially expressed on neutrophilic granulocytes, inflammatory monocytes, and some populations of dendritic cells (13, 14, 19–21).

Here, we found that large numbers of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells accumulated and persisted in tissues of mice infected with *S. Typhimurium*. We characterized these cells and found that recruitment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> but not CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells from the bone marrow depended on chemokine receptor 2 (CCR2). Splenic CD11b<sup>+</sup>

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Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells differentiated into macrophage-like cells following *ex vivo* culture and could present antigen to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells *in vitro*. However, robust proliferation of the T cells was observed only when the ability of the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells to produce NO was blocked. CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells recruited in response to *S. Typhimurium* infection could also present antigen to T cells *in vivo*, but increasing the number of these cells by adoptive transfer did not cause a corresponding increase in T cell response. Thus, CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> immature myeloid cells provide a balance of immunosuppressive and protective functions in the host response to *S. Typhimurium*. The tipping of this balance may be an important factor influencing the outcome of infection.

## MATERIALS AND METHODS

**Bacteria.** *S. Typhimurium* strain IR715 (22), which is a spontaneous, nalidixic acid-resistant derivative of *S. Typhimurium* strain 14028 (American Type Culture Collection), was used as the wild-type strain. Bacteria were grown aerobically for 12 to 18 h at 37°C in 3 ml of Luria-Bertani (LB) broth (250 rpm) or on LB agar plates using standard microbiological techniques.

**Mice.** C57BL/6J and 129X1/SvJ mice (both H-2<sup>b</sup> haplotype) were purchased from The Jackson Laboratory. These strains of mice have been used as model hosts to study acute and persistent salmonellosis, respectively (4). F1 (C57BL/6J × 129X1/SvJ) hybrid mice, which can accept cells from either C57BL/6J or 129X1/SvJ mice, were bred at Stony Brook University, Division of Laboratory Animal Resources. B6.129S4-Ccr2<sup>tm1Jc/J</sup> (Ccr2<sup>-/-</sup>), C57BL/6-Tg(TcrαTcrβ)1100Mjb/J (OT-I), and B6.Cg-Tg(TcrαTcrβ)425Cbn/J (OT-II) mice (all H-2<sup>b</sup> haplotype) were purchased from The Jackson Laboratory and bred at Stony Brook University, Division of Laboratory Animal Resources. Ccr2<sup>-/-</sup> mice lack chemokine receptor CCR2. OT-I mice are T-cell-receptor (TCR) transgenic mice in the C57BL/6J strain background that produce CD8<sup>+</sup> T cells specific for amino acid residues 257 to 264 of chicken egg ovalbumin (OVA<sub>257-264</sub>) bound to class I major histocompatibility complex (MHC) (H-2K<sup>b</sup>). OT-II mice are TCR transgenic mice in the C57BL/6J strain background that produce CD4<sup>+</sup> T cells specific for amino acid residues 323 to 339 of chicken egg ovalbumin (OVA<sub>323-339</sub>) bound to class II MHC (I-A<sup>b</sup>). The Institutional Animal Care and Use Committee at Stony Brook University approved all procedures and experiments using mice.

**Mouse infections.** Mouse infections were performed using naive, 8- to 12-week-old sex-matched C57BL/6J, 129X1/SvJ, and Ccr2<sup>-/-</sup> mice. Briefly, mice were inoculated intragastrically with *S. Typhimurium* (5 × 10<sup>7</sup> CFU for C57BL/6J and Ccr2<sup>-/-</sup> mice and 5 × 10<sup>8</sup> CFU for 129X1/SvJ mice) suspended in 0.1 ml of phosphate-buffered saline (PBS). To improve the consistency of intragastric infections, food (but not water) was removed 6 to 8 h prior to inoculation. Immediately following inoculation, food was provided *ad libitum*. Tenfold serial dilutions of the inoculum were plated on LB agar to confirm the inoculum titer. At indicated times after inoculation, target organs (i.e., spleen, liver, mesenteric lymph nodes, and bone marrow) were harvested and processed to obtain single-cell suspensions. At each time point, we controlled for the effect of age by preparing samples from two age-matched mice left uninfected. At the end of the experiment, the results were combined and reported as the uninfected control. Bacterial loads were determined by lysing the cells with Triton X-100 (0.05%) and plating for CFU on LB agar containing nalidixic acid (50 μg/ml). Mice infected with *S. Typhimurium* were euthanized when moribund or at the termination of the experiment.

**Cell staining and analysis by flow cytometry.** Conjugated monoclonal antibodies and reagents described here were purchased from BioLegend unless indicated otherwise. Routinely, cells were stained in the presence of Fc block (anti-mouse CD16/32 antibody; clone 93) using anti-mouse antibodies specific for Gr-1 (clone RB6-8C5), Ly6C (clone HK1.4), Ly6G (clone 1A8), CD11b (clone M1/70), F4/80 (clone CI:A3-1), and I-A<sup>b</sup>

(clone AF6-120.1). In T cell assays, cells were stained in the presence of Fc block using anti-mouse antibodies specific for CD4 (clone RM4-5), CD8β (clone YTS156.7.7), and CD90.2 (clone 30-H12) to identify T cells; anti-mouse antibodies specific for Vα2 (clone B20.1) and Vβ5.1/5.2 (clone MR9-4; BD Biosciences) to identify the T cell receptor expressed by OT-I and OT-II T cells; and anti-mouse antibody specific for CD69 (clone H1.2F3) to measure T cell activation. Data were acquired and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) with BD CellQuestPro (BD Biosciences) and FlowJo (Tree Star) software or a BD FACSAria cell sorter with BD FACSDiva software (BD Biosciences).

**CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cell purification and analysis.** For all experiments that used purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells, splenocytes harvested from 129X1/SvJ mice infected with *S. Typhimurium* for 21 to 28 days were used as a source of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells. Following treatment of the splenocytes with ACK lysing buffer (0.15 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>-EDTA) to lyse red blood cells, magnetic cell separation (MACS) technology (Miltenyi Biotec) was used to enrich for CD11b<sup>+</sup> cells. Throughout this process and all other manipulations of the cells, antibiotics (i.e., penicillin, streptomycin, and gentamicin) were present to kill all bacteria. Enriched populations of CD11b<sup>+</sup> cells were stained in the presence of Fc block using antibodies specific for CD11b, Ly6C, and Ly6G, as described above. A BD FACSAria cell sorter with BD FACSDiva software was used to purify the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells. In preparation for morphological analysis, 1 × 10<sup>5</sup> purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> or CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells were centrifuged onto glass microscope slides that were subsequently air dried. The cells were then fixed using methanol, stained using Reastain Quick-Diff (Reagen), and visualized by light microscopy.

**Nitrite assay.** Purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells were seeded into flat-bottom 96-well tissue culture plates at 1 × 10<sup>6</sup> cells per well. After 16 h of incubation at 37°C in 5% CO<sub>2</sub>, we measured the levels of nitrite in supernatant using the Griess reagent system (Promega).

**Ex vivo culture of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells.** Purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells were seeded into 24-well tissue culture plates at 1 × 10<sup>5</sup> cells per well using medium formulated for the *ex vivo* culture of bone marrow-derived macrophages (Dulbecco's modified Eagle medium [DMEM] with GlutaMAX-1 [Invitrogen] supplemented with 20% heat-inactivated fetal bovine serum [Atlanta Biologicals], 20% L-cell conditioned medium [a source of macrophage colony-stimulating factor {M-CSF}], 0.2 M L-Gln, 0.1 M sodium pyruvate, and 1% penicillin-streptomycin). After 7 days of *ex vivo* culture at 37°C in 5% CO<sub>2</sub>, the cells were visualized by light microscopy and analyzed by flow cytometry. As a positive control, bone marrow-derived macrophages were cultured from naive 129X1/SvJ mice, as described previously (12, 23).

**T cell enrichment and T cell assays.** Splenocytes harvested from naive OT-I, OT-II, and 129X1/SvJ mice were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer, MACS technology was used to enrich for CD90.2<sup>+</sup> T cells. Enriched populations of T cells were suspended in RP-10 medium (RPMI 1640 medium [Invitrogen] supplemented with 10% fetal bovine serum, 0.2 M L-Gln, 0.1 M HEPES, 50 μM 2-mercaptoethanol [2-ME], and 1% penicillin-streptomycin), labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen), and used in T cell assays. CFSE is a cell-permeant fluorescent dye that, once taken up, is retained and distributed evenly among daughter cells with each round of cell division, resulting in a quantum reduction in cell fluorescence that can be measured by flow cytometry (24).

In assays aimed at measuring antigen-induced T cell proliferation, purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells were mock treated or coated with 5 nM OVA<sub>257-264</sub> or 5 μM OVA<sub>323-339</sub> peptide (Bio-Synthesis), suspended in RP-10, and seeded into round-bottom 96-well tissue culture plates at 5 × 10<sup>4</sup> cells per well. Where indicated, the mock-treated or peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells were fixed with 2% paraformaldehyde.

hyde (Sigma) and treated with 0.2 M L-lysine (Sigma), as described previously (25). CFSE-labeled OT-I ( $V\alpha 2^+ V\beta 5^+ CD8\beta^+$ ) or OT-II ( $V\alpha 2^+ V\beta 5^+ CD4^+$ ) T cells were then added to the  $CD11b^+ Ly6C^{hi} Ly6G^-$  cells at indicated ratios. After 4 days of incubation at  $37^\circ C$  in 5%  $CO_2$ , cells were harvested, stained, and analyzed by flow cytometry.

In assays aimed at measuring polyclonal T cell proliferation, CFSE-labeled 129X1/SvJ T cells were suspended in RP-10, seeded into round-bottom 96-well tissue culture plates coated with 3  $\mu g/ml$  anti-mouse CD3 $\epsilon$  antibody (clone 145-2C11; BioLegend) at  $5 \times 10^4$  cells per well, and cultured in the presence of 5  $\mu g/ml$  anti-mouse CD28 antibody (clone E18; BioLegend). Purified  $CD11b^+ Ly6C^{hi} Ly6G^-$  cells were then added to the T cells at a 10:1 or 1:1 ratio. Where indicated, the inducible nitric oxide synthase (iNOS) inhibitor 1400W (Sigma) was added to the cultures at a final concentration of 200  $\mu M$ . After 4 days of incubation at  $37^\circ C$  in 5%  $CO_2$ , cells were harvested, stained, and analyzed by flow cytometry.

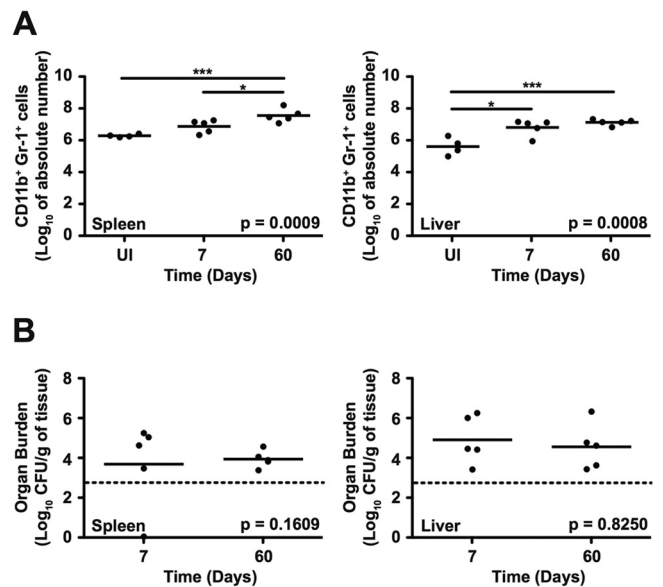
In assays aimed at measuring antigen-induced T cell activation *in vivo*, the equivalent of  $1 \times 10^6$  splenic OVA<sub>257–264</sub>-specific  $CD8^+$  T cells from naive OT-I mice were adoptively transferred into naive F1 (C57BL/6J  $\times$  129X1/SvJ) mice by intravenous injection. The number of OT-I splenocytes transferred was based on the frequency of  $V\alpha 2^+ V\beta 5^+ CD8\beta^+$  cells. One day later, recipient mice received  $1 \times 10^6$  or  $2 \times 10^6$  purified, OVA<sub>257–264</sub> peptide-coated  $CD11b^+ Ly6C^{hi} Ly6G^-$  cells by intravenous injection. As a positive control, recipient mice received  $1 \times 10^6$  or  $2 \times 10^6$  OVA<sub>257–264</sub> peptide-coated bone marrow-derived macrophages. As a negative control, recipient mice received 200  $\mu l$  of PBS. One day after the second adoptive transfer, splenocytes were harvested; stained with anti-mouse antibodies specific for  $V\alpha 2$ ,  $V\beta 5$ ,  $CD8\beta$ , and  $CD69$ ; and analyzed by flow cytometry.

**Statistical analysis.** Statistical analysis was performed using Prism 5.0b (GraphPad Software). Data were analyzed using two-tailed, paired Student's *t* test, one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison posttest, or two-way ANOVA with Bonferroni's posttest; *P* values of  $<0.05$  were considered to be statistically significant. Asterisks in the figures indicate statistically significant differences (\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).

## RESULTS

**Large numbers of  $CD11b^+ Gr-1^+$  cells accumulate in tissues of mice infected with *S. Typhimurium*.** To dissect innate immune mechanisms that control spread and growth of *S. Typhimurium*, we characterized cellular responses and determined bacterial loads in spleens and livers of 129X1/SvJ mice inoculated intragastrically with  $5 \times 10^8$  CFU of *S. Typhimurium*. After 7 days of infection, we found more  $CD11b^+ Gr-1^+$  cells in spleens and livers of mice infected with *S. Typhimurium* than in spleens and livers of mice left uninfected (Fig. 1A; see also Fig. S1A in the supplemental material). After 60 days of infection, a long-term time point used here to assess bacterial persistence (Fig. 1B), absolute numbers and percentages of  $CD11b^+ Gr-1^+$  cells in spleen and liver had increased even further (28-fold and 20-fold, respectively) (Fig. 1A; see also Fig. S1A). Similar results were obtained when we characterized cellular responses in C57BL/6J mice inoculated intragastrically with  $5 \times 10^7$  CFU of *S. Typhimurium* (see Fig. S1B). Thus, large numbers of  $CD11b^+ Gr-1^+$  cells accumulate in tissues of mice infected with *S. Typhimurium*.

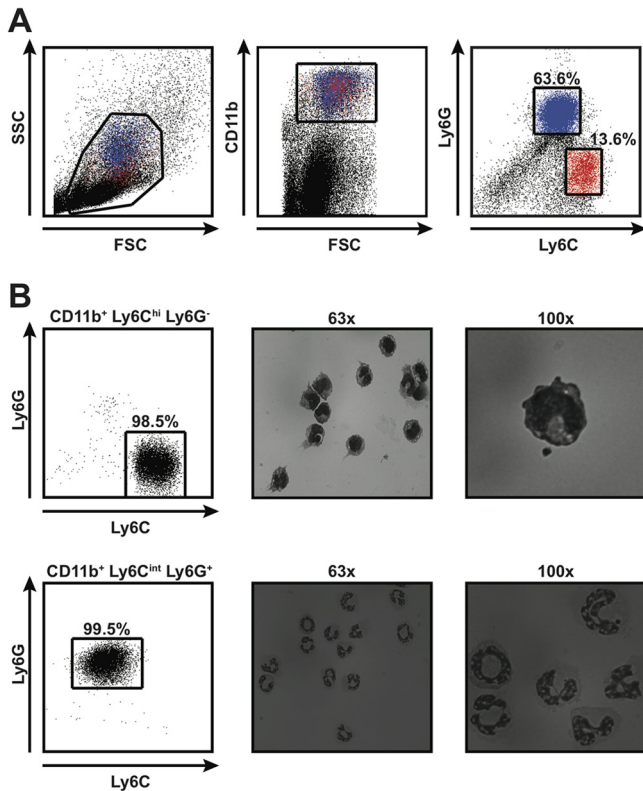
**$CD11b^+ Gr-1^+$  cells that accumulate in tissues of mice infected with *S. Typhimurium* exhibit phenotypic and morphological heterogeneity.**  $CD11b^+ Gr-1^+$  cells are a heterogeneous population that can be further divided based on expression of  $Ly6C$  and  $Ly6G$ , two isoforms of  $Gr-1$  that are differentially expressed on the surface of neutrophilic granulocytes, inflammatory monocytes, and some populations of dendritic cells (13, 14, 19–21). To define the phenotypic and morphological heterogeneity of



**FIG 1** Large numbers of  $CD11b^+ Gr-1^+$  cells accumulate in tissues of mice infected with *S. Typhimurium*. (A) Absolute numbers of  $CD11b^+ Gr-1^+$  cells in spleens (left) and livers (right) of 129X1/SvJ mice ( $n = 4$  to 5 per group per time point) left uninfected (UI) or infected for 7 or 60 days with *S. Typhimurium*. (B) Corresponding bacterial loads per gram of spleen (left) and liver (right) tissue. Dashed lines denote limits of detection. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using a one-way ANOVA with Bonferroni's multiple-comparison posttest; *P* values of  $<0.05$  were considered to be statistically significant. Asterisks indicate statistically significant differences (\*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ ). See also Fig. S1 in the supplemental material.

$CD11b^+ Gr-1^+$  cells in the host response to *S. Typhimurium*, we further characterized the  $CD11b^+ Gr-1^+$  cells present in spleens of 129X1/SvJ mice infected with *S. Typhimurium* for 21 to 28 days. We found the  $CD11b^+ Gr-1^+$  cells to be a heterogeneous population that could be divided into  $CD11b^+ Ly6C^{hi} Ly6G^-$  and  $CD11b^+ Ly6C^{int} Ly6G^+$  cells (Fig. 2A). Furthermore, we found that purified  $CD11b^+ Ly6C^{hi} Ly6G^-$  cells exhibited a mononuclear morphology, whereas purified  $CD11b^+ Ly6C^{int} Ly6G^+$  cells exhibited a polymorphonuclear or band-shaped nuclear morphology (Fig. 2B). Thus,  $CD11b^+ Gr-1^+$  cells in the host response to *S. Typhimurium* are characterized by phenotypic and morphological heterogeneity.

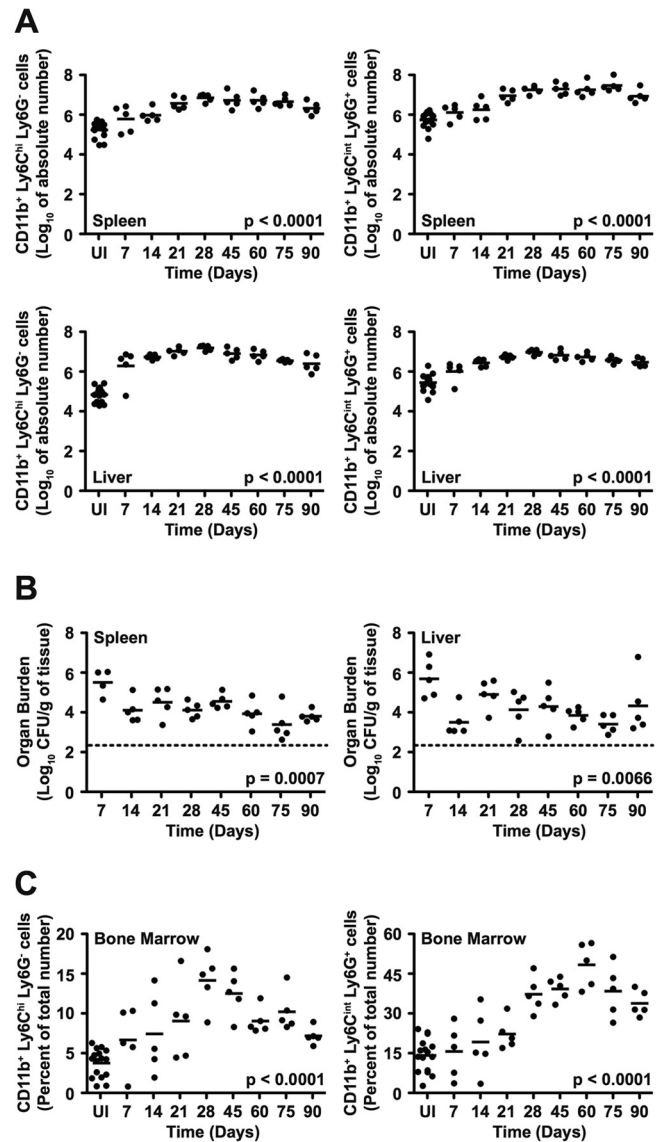
**$CD11b^+ Ly6C^{hi} Ly6G^-$  and  $CD11b^+ Ly6C^{int} Ly6G^+$  cells accumulate and persist in tissues of mice infected with *S. Typhimurium*.** To determine the kinetics with which  $CD11b^+ Ly6C^{hi} Ly6G^-$  and  $CD11b^+ Ly6C^{int} Ly6G^+$  cells respond during infection, we further characterized cellular responses in 129X1/SvJ mice inoculated with *S. Typhimurium*. After 7 days of infection, we found more  $CD11b^+ Ly6C^{hi} Ly6G^-$  and  $CD11b^+ Ly6C^{int} Ly6G^+$  cells in spleens and livers of mice infected with *S. Typhimurium* than in spleens and livers of mice left uninfected (Fig. 3A; see also Fig. S2A in the supplemental material). Both absolute numbers and percentages of  $CD11b^+ Ly6C^{hi} Ly6G^-$  and  $CD11b^+ Ly6C^{int} Ly6G^+$  cells increased dramatically over time (34- and 28-fold increases in absolute numbers in spleen and 172- and 23-fold increases in absolute numbers in liver, respectively, after 28 days of infection), plateaued at around day 45 after inoculation, and persisted at peak or near peak levels over a period of 90 days



**FIG 2** CD11b<sup>+</sup> Gr-1<sup>+</sup> cells that accumulate in tissues of mice infected with *S. Typhimurium* exhibit phenotypic and morphological heterogeneity. (A) Flow cytometric analysis of splenocytes harvested from 129X1/SvJ mice infected for 28 days with *S. Typhimurium*. Live splenocytes (left) that expressed CD11b (middle) could be divided into CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells (right). Red and blue colors indicate CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells, respectively. Numbers refer to CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells as percentages of live, CD11b<sup>+</sup> cells. FSC, forward scatter; SSC, side scatter. (B) Morphological analysis of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells purified by fluorescence-activated cell sorting. The purity of the cells was consistently greater than 98% (left). Purified cells were stained with Reastain Quick-Diff and visualized by light microscopy at ×63 (middle) and ×100 (right) magnifications. Numbers refer to CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells as percentages of live cells. Data are representative of six independent experiments.

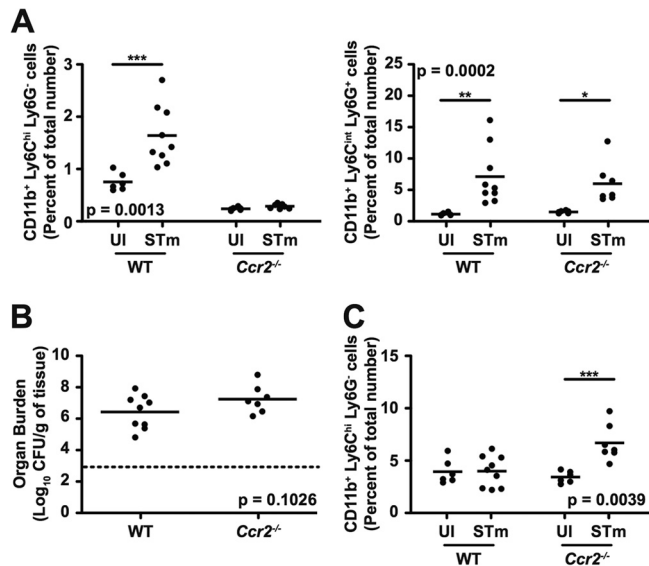
(Fig. 3A; see also Fig. S2A). Similar results were obtained when we characterized CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cell responses in mesenteric lymph nodes (see Fig. S2B). We recovered substantial numbers of *S. Typhimurium* bacteria from spleens and livers of infected mice at every time point (Fig. 3B), indicating that the response of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells correlated with bacterial persistence. The kinetics with which CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells responded in bone marrow were similar to those in spleen and liver (Fig. 3C). Thus, accumulation and persistence of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells in peripheral tissues of mice infected with *S. Typhimurium* could be the result of increased output from this generative site.

**CCR2 is required for the recruitment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells from the bone marrow to systemic sites of *S. Typhimurium* infection.** CCR2 is critical for emigration of monocyte precursors from the bone marrow (14, 15). To define the role of CCR2 in innate immune defense against *S. Typhimurium*, we



**FIG 3** CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells accumulate and persist in tissues of mice infected with *S. Typhimurium*. (A) Absolute numbers of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells (left) and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells (right) in spleens (top) and livers (bottom) of 129X1/SvJ mice ( $n = 5$  per group per time point) left uninfected (UI) or infected for up to 90 days with *S. Typhimurium*. (B) Bacterial loads per gram of spleen (left) and liver (right) tissue. (C) Percentages of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells (left) and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells (right) in bone marrow. Dashed lines denote limits of detection. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using a one-way ANOVA;  $P$  values of  $<0.05$  were considered to be statistically significant. See also Fig. S2 in the supplemental material.

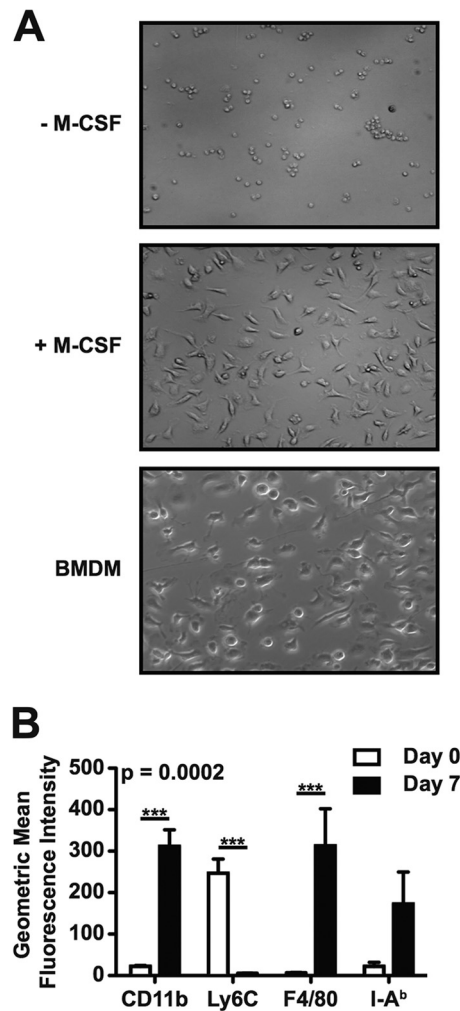
characterized cellular responses in wild-type (WT) and *Ccr2*<sup>-/-</sup> C57BL/6J mice inoculated intragastrically with  $5 \times 10^7$  CFU of *S. Typhimurium*. After 7 days of infection, we observed significant accumulation of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells in spleens of WT but not *Ccr2*<sup>-/-</sup> mice (Fig. 4A, left panel). As expected for granulocyte precursors, which do not require CCR2 for emigration from the bone marrow (15), we observed significant and comparable accumulation of CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells in spleens of both WT and *Ccr2*<sup>-/-</sup> mice (Fig. 4A, right panel). We recovered similar



**FIG 4** CCR2 is required for the recruitment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells from the bone marrow to systemic sites of *S. Typhimurium* infection. (A) Percentages of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells (left) and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells (right) in spleens of WT and *Ccr2*<sup>-/-</sup> mice (*n* = 6 to 9 per group) left uninfected (UI) or infected for 7 days with *S. Typhimurium* (STm). (B) Bacterial loads per gram of spleen tissue. (C) Percentages of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells in bone marrow. The dashed line denotes the limit of detection. Data show mean with spread and are cumulative of two independent experiments. Data were analyzed using a two-way ANOVA with Bonferroni's post-test (A and C) or using two-tailed, paired Student's *t* test (B); *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (\*\*\*, *P* < 0.001; \*\*, *P* < 0.01; \*, *P* < 0.05).

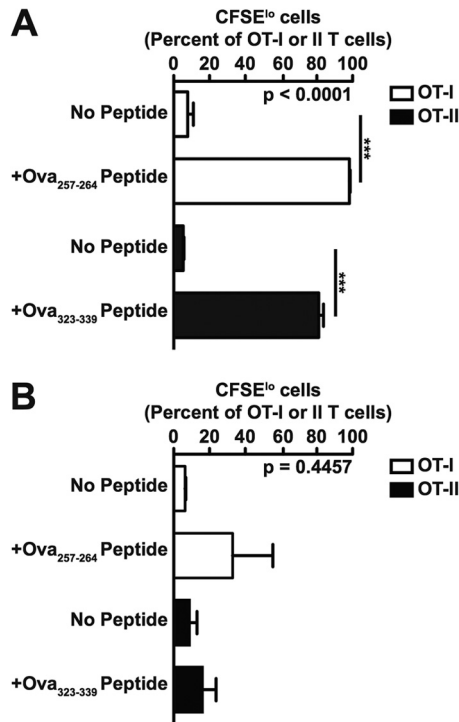
numbers of *S. Typhimurium* bacteria from spleens of infected WT and *Ccr2*<sup>-/-</sup> mice (Fig. 4B), indicating that the lack of accumulation of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells in spleens of infected *Ccr2*<sup>-/-</sup> mice was not due to a difference in bacterial load. Consistent with a role for CCR2 in emigration of monocyte precursors from the bone marrow (14), we observed significant accumulation of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells in bone marrow of infected *Ccr2*<sup>-/-</sup> but not WT mice (Fig. 4C). Thus, CCR2 is required for the recruitment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells from the bone marrow to systemic sites of *S. Typhimurium* infection.

**CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* can differentiate into macrophages following *ex vivo* culture.** Next, we examined the ability of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells purified from spleens of 129X1/SvJ mice infected with *S. Typhimurium* to differentiate *ex vivo*. After 7 days of *ex vivo* culture in the presence of L-cell conditioned medium, a source of M-CSF, we found that the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> but not CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells had differentiated into adherent cells that exhibited a macrophage-like morphology (Fig. 5A and data not shown). The differentiated cells expressed increased levels of surface CD11b and F4/80 but had lost expression of surface Ly6C (Fig. 5B). The differentiated cells also expressed increased levels of surface class II MHC (I-A<sup>b</sup>), but the observed difference did not reach statistical significance (Fig. 5B). Collectively, these results indicate that the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells that accumulate in spleens of mice infected with *S. Typhimurium* are immature myeloid cells capable of differentiating into macrophages.



**FIG 5** CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* can differentiate into macrophage-like cells following *ex vivo* culture. (A) Light microscopy images of purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells cultured *ex vivo* for 7 days in the absence (top) or presence (middle) of M-CSF. Cultures of bone marrow-derived macrophages (BMDM; bottom) were used as a control. (B) Flow cytometric analysis of purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells (white bars; day 0) or purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells cultured *ex vivo* for 7 days in the presence of M-CSF (black bars; day 7). Analysis was performed to detect expression of surface CD11b, Ly6C, F4/80, and class II MHC (I-A<sup>b</sup>). Data are representative of (A), or show the mean with the standard error of the mean from (B), three independent experiments. Data were analyzed using a two-way ANOVA with Bonferroni's posttest; *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (\*\*\*, *P* < 0.001).

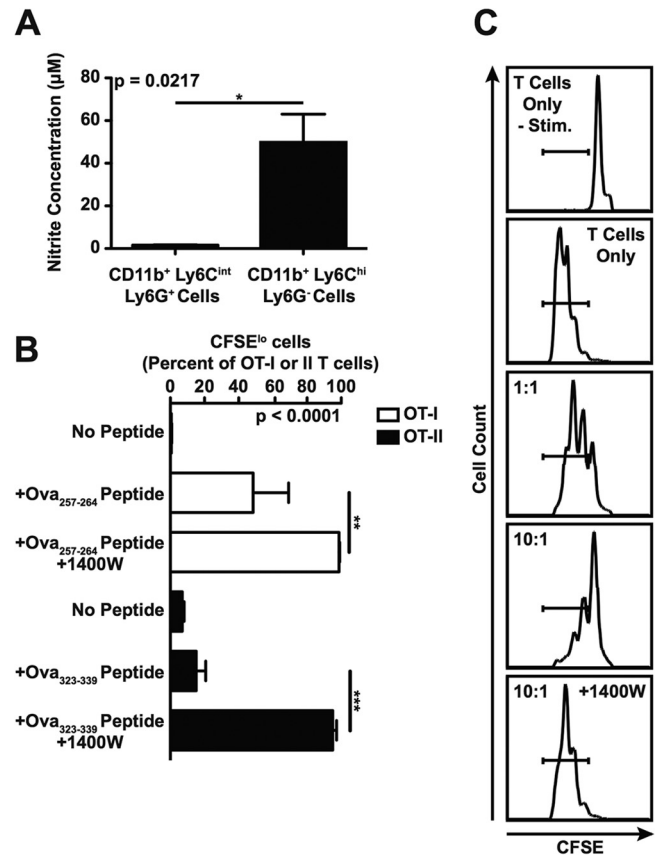
**CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* can present antigen to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.** Given that CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* could differentiate into macrophage-like cells (Fig. 5), we characterized the ability of these immature myeloid cells to present antigen to T cells *in vitro*. T cells enriched from spleens of naive OT-I mice, which produce CD8<sup>+</sup> T cells specific for amino acid residues 257 to 264 of chicken egg ovalbumin (OVA<sub>257-264</sub>) bound to class I MHC (H-2K<sup>b</sup>), or OT-II mice, which produce CD4<sup>+</sup> T cells specific for amino acid residues 323 to 339 of chicken egg ovalbumin



**FIG 6** CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* can present antigen to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Proliferation of CFSE-labeled OT-I T cells (white bars) or OT-II T cells (black bars) cultured with paraformaldehyde-fixed (A) or live (B), purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells mock treated (No Peptide) or coated with OVA<sub>257-264</sub> or OVA<sub>323-339</sub> peptide. The ratio of antigen-presenting cells to responder cells was 5:1. Data show means with standard errors of the means from three independent experiments. Data were analyzed using one-way ANOVA with Bonferroni's multiple-comparison posttest; *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (\*\*\*, *P* < 0.001).

(OVA<sub>323-339</sub>) bound to class II MHC (I-A<sup>b</sup>), were labeled with CFSE and used as responder cells. CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of 129X1/SvJ mice infected with *S. Typhimurium* were mock treated or coated with OVA<sub>257-264</sub> or OVA<sub>323-339</sub> peptide, fixed with paraformaldehyde, and used as antigen-presenting cells. Responder T cells and antigen-presenting cells were mixed at a 5:1 ratio and seeded into tissue culture plates. After 4 days of incubation, we found that T cells cultured with peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells had proliferated extensively compared to T cells cultured with mock-treated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells (Fig. 6A). However, significant proliferation of the T cells was not observed when live antigen-presenting cells were used (Fig. 6B). Thus, the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> immature myeloid cells that accumulate and persist in spleens of mice infected with *S. Typhimurium* can present antigen to T cells when fixed but produce an inhibitor of T cell proliferation when alive.

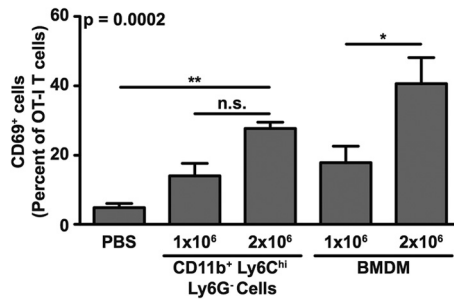
**CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* can inhibit T cell proliferation via a NO-dependent mechanism.** Early studies linked NO-producing macrophage precursors to immunosuppression in salmonellosis (16). Therefore, we examined the ability of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells purified from spleens of mice infected with *S. Typhimurium* to produce NO.



**FIG 7** CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of mice infected with *S. Typhimurium* can inhibit T cell proliferation via a NO-dependent mechanism. (A) Production of nitrite by purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells as measured using the Griess reagent system. (B) Proliferation of CFSE-labeled OT-I T cells (white bars) or OT-II T cells (black bars) cultured with purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells mock treated (No Peptide) or coated with OVA<sub>257-264</sub> or OVA<sub>323-339</sub> peptide. The ratio of antigen-presenting cells to responder cells was 10:1. Where indicated, the iNOS inhibitor 1400W was added to the cultures. (C) Proliferation of CFSE-labeled 129X1/SvJ T cells cultured in the absence or presence of purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> bystander cells. Proliferation of the T cells was induced using anti-mouse CD3ε and anti-mouse CD28 antibody, except where noted (-Stim.). The ratio of bystander cells to responder cells was 1:1 or 10:1. Where indicated, the iNOS inhibitor 1400W was added to the cultures. Data show means with standard errors of the means from (A and B), or are representative of (C), at least three independent experiments. Data were analyzed using two-tailed, paired Student's *t* test (A) or using a one-way ANOVA with Bonferroni's multiple-comparison posttest (B); *P* values of <0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (\*\*\*, *P* < 0.001; \*\*, *P* < 0.01; \*, *P* < 0.05). See also Fig. S3 in the supplemental material.

After 16 h of *ex vivo* culture, we found high levels of nitrite in the supernatants of the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> but not CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells (Fig. 7A), indicating that large amounts of NO were produced by the former but not the latter population of cells.

To define the impact of NO production by the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells on the ability of T cells to proliferate, we added the selective iNOS inhibitor 1400W to cultures of responder T cells mixed with OVA peptide-coated antigen-presenting cells, as described above. After 4 days of incubation, we found that T cells cultured with peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells and the selective iNOS inhibitor 1400W had proliferated significantly



**FIG 8** CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells recruited in response to *S. Typhimurium* infection can modulate T cell function *in vivo*. Activation of adoptively transferred OT-I T cells in F1 (C57BL/6) × 129X1/SvJ mice (cumulative total of  $n = 4$  per group) that received OVA<sub>257–264</sub> peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of 129X1/SvJ mice infected with *S. Typhimurium*. Activation of OT-I T cells was measured by analyzing expression of surface CD69. Recipient mice that received PBS or OVA<sub>257–264</sub> peptide-coated bone marrow-derived macrophages were used as controls. Cumulative data from three independent experiments show means with standard errors of the means and were analyzed using a one-way ANOVA with Bonferroni's multiple-comparison posttest;  $P$  values of  $<0.05$  were considered to be statistically significant. Asterisks indicate statistically significant differences (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; n.s., not significant).

more than had T cells cultured with peptide-coated or mock-treated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells only (Fig. 7B). Similar results were obtained when we examined the ability of purified CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells, most of which are uninfected, to suppress polyclonal T cell proliferation through a bystander effect (Fig. 7C; see also Fig. S3 in the supplemental material). Thus, the CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> immature myeloid cells that accumulate and persist in tissues of mice infected with *S. Typhimurium* can inhibit T cell proliferation via a NO-dependent mechanism. Furthermore, these results indicate that the accumulation and persistence of NO-producing CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> immature myeloid cells in peripheral tissues of mice infected with *S. Typhimurium* could lead to a bystander effect responsible for the delay in onset of protective immune responses.

**CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells recruited in response to *S. Typhimurium* infection can modulate T cell function *in vivo*.** Next, we used an adoptive transfer approach to determine the ability of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells recruited in response to *S. Typhimurium* infection to modulate T cell function *in vivo*. Splenocytes from naive OT-I mice were used as a source of T cells. We adoptively transferred  $1 \times 10^6$  OT-I T cells into naive F1 (C57BL/6) × 129X1/SvJ mice. One day later, recipient mice received  $1 \times 10^6$  or  $2 \times 10^6$  OVA<sub>257–264</sub> peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells purified from spleens of 129X1/SvJ mice infected with *S. Typhimurium*. Recipient mice that received PBS or OVA<sub>257–264</sub> peptide-coated bone marrow-derived macrophages were used as controls. One day after the second adoptive transfer, splenocytes were harvested, stained, and analyzed to determine the level of OT-I T cell activation. We found that the percentages of activated OT-I T cells in spleens of mice that received OVA<sub>257–264</sub> peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells were significantly higher than the percentage of activated OT-I T cells in spleens of mice that received PBS (Fig. 8). However, no significant increase in the percentage of activated OT-I T cells was observed in spleens of mice that received  $2 \times 10^6$  instead of  $1 \times 10^6$  OVA<sub>257–264</sub> peptide-coated CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells (Fig. 8). In contrast, we found

that a 2-fold increase in the number of OVA<sub>257–264</sub> peptide-coated bone marrow-derived macrophages transferred resulted in a corresponding 2-fold increase in the percentage of activated OT-I T cells in spleen (Fig. 8). Thus, CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells recruited in response to *S. Typhimurium* infection can present antigen to T cells *in vivo*, but as their numbers in the periphery increase, there does not appear to be a corresponding increase in the response of T cells. We interpret these results to suggest that accumulation and persistence of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells beyond a certain threshold level may cause immunosuppression.

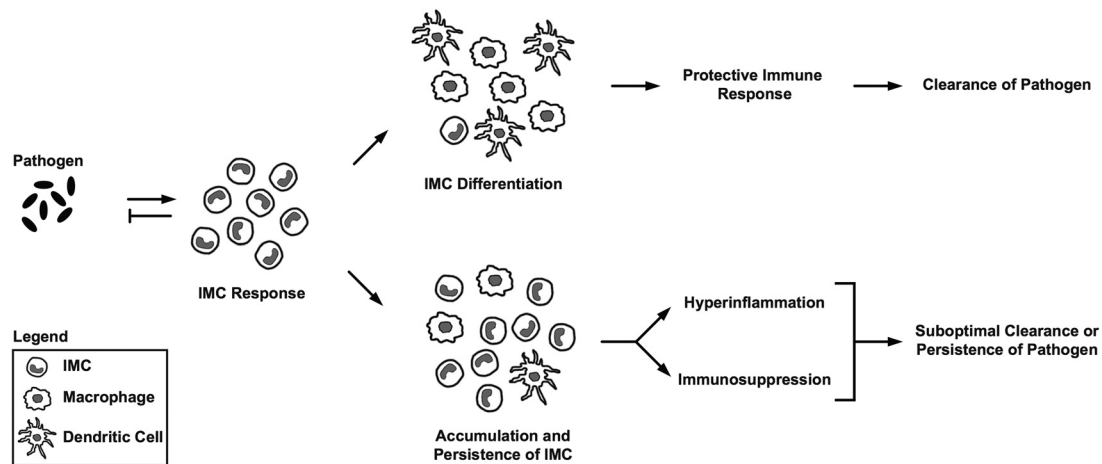
## DISCUSSION

We have found that large numbers of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells accumulate and persist in tissues of mice infected with *S. Typhimurium*. Furthermore, we have found that CCR2 is critical for the recruitment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells from the bone marrow to systemic sites of *S. Typhimurium* infection, where these cells may regulate CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses via antigen presentation and NO production. Our results provide an understanding of a mechanism by which *S. Typhimurium* may induce acute immunosuppression and delay onset of protective immune responses. An immediate implication of our results is that, while phagocytes are essential for control of *S. Typhimurium*, accumulation and persistence of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> phagocyte precursors in infected tissues could lead to a buildup of NO, a potent antimicrobial effector molecule that can also cause suppression of T cell function (26–28). We propose a model in which NO-producing CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> immature myeloid cells provide protective functions in the host response to infection, where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease (Fig. 9).

Consistent with a role for CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells in *S. Typhimurium*-induced inhibition of T cell responses, immature myeloid cells that have differentiated into inflammatory monocytes have been implicated recently in cytomegalovirus-induced suppression of antiviral CD8<sup>+</sup> T cell responses (29). Furthermore, sustained expansion of immature myeloid cells in chronic but not acute infection with lymphocytic choriomeningitis virus has been linked to inhibition of antiviral CD8<sup>+</sup> T cell responses (30). Thus, immature myeloid cells provide a balance of protective and immunosuppressive functions in the host response to infection.

The accumulation and persistence of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> and CD11b<sup>+</sup> Ly6C<sup>int</sup> Ly6G<sup>+</sup> cells in tissues of mice infected with *S. Typhimurium* could be the result of sustained inflammation, hyperinflammation, or failure to differentiate into protective cell populations such as macrophages, dendritic cells, and granulocytes. CD11b<sup>+</sup> Gr-1<sup>+</sup> cells are the predominant population of cells targeted by *S. Typhimurium* type III secretion *in vivo* and contain most of the total number of intracellular bacteria recovered from spleen (18). However, the majority of splenic immature myeloid cells are not directly infected with *S. Typhimurium* (see Fig. S3 in the supplemental material), and therefore, it is unknown if *S. Typhimurium* can directly inhibit differentiation of immature myeloid cells through a mechanism that would require targeting of these cells by type III secretion.

Suppression of T cell function by CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cells occurs through the iNOS metabolic pathway and may be exploited by *S. Typhimurium* to avoid clearance. Consistent with this notion, the kinetics of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>-</sup> cell accumula-



**FIG 9** Model of the role of  $CD11b^+ Ly6C^{hi} Ly6G^-$  immature myeloid cells (IMC) in the host response to infection. We propose a model in which NO-producing  $CD11b^+ Ly6C^{hi} Ly6G^-$  IMC provide protective functions in the host response to infection (top), where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease (bottom). For further explanation, see the text.

tion and persistence correspond to the delay in onset and dampening of T cell responses to *S. Typhimurium*, which peak in weeks 3 to 4 after infection (9, 12, 31). Furthermore, early studies linked NO-producing macrophage precursors to *S. Typhimurium*-induced immunosuppression and protective immunity against *S. Typhimurium* to diminished expansion of  $CD11b^+ Gr-1^+$  cells (16, 17). Several recent studies have associated  $CD11b^+ Gr-1^+$  cells with functional immunosuppression in the host response to infection (29, 30, 32–36), and some have implicated NO production by these cells in the suppression of T cell function. Thus, a role for NO-producing immature myeloid cells in mediating immunosuppression may be a common feature of many types of infections, particularly persistent or chronic infections.

The  $CD11b^+ Ly6C^{hi} Ly6G^-$  cells that accumulate and persist in tissues of mice infected with *S. Typhimurium* resemble mononuclear myeloid-derived suppressor cells (MDSC), which have been associated with immunosuppression in cancer and, more recently, infection (37, 38). Direct roles of iNOS and arginase-1 in the suppressive activity of mononuclear MDSC are well established, whereas reactive oxygen species and peroxynitrite have emerged more recently as factors that contribute to the suppressive activity of MDSC (37, 38). Thus, production of NO, which can freely diffuse across membranes, may be only one of the immunomodulatory factors produced by  $CD11b^+ Ly6C^{hi} Ly6G^-$  cells in the host response to *S. Typhimurium*.

In conclusion, our work provides an immunologic basis for a host-directed component that can exacerbate *S. Typhimurium* infection by inhibiting T cell responses. Historically, immature myeloid cells are considered essential for the protective host response to infection (15). Here, we establish that  $CD11b^+ Ly6C^{hi} Ly6G^-$  immature myeloid cells provide a balance of protective and immunosuppressive functions in the host response to *S. Typhimurium*. The tipping of this balance may be an important factor influencing the outcome of infection. Thus,  $CD11b^+ Ly6C^{hi} Ly6G^-$  immature myeloid cells could prove to be a promising target for the development of innovative, broad-spectrum therapeutic approaches to overcome infection.

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