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*J Immunol* 2007; 178:5789-5801;

doi: 10.4049/jimmunol.178.9.5789

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Monocyte Recruitment, Activation, and Function in the Gut-Associated Lymphoid Tissue during Oral Salmonella Infection

Anna Rydström and Mary Jo Wick

Neutrophils, monocytes, and dendritic cells (DC) are phenotypically and functionally related phagocytes whose presence in infected tissues is critical to host survival. Their overlapping expression pattern of surface molecules, the differentiation capacity of monocytes, and the presence of monocyte subsets underscores the complexity of understanding the role of these cells during infection. In this study we use five- to seven-color flow cytometry to assess the phenotype and function of monocytes recruited to Peyer’s patches (PP) and mesenteric lymph nodes (MLN) after oral Salmonella infection of mice. The data show that CD68intGr-1int (intermediate) monocytes, along with CD68intGr-1int neutrophils, rapidly accumulate in PP and MLN. The monocytes have increased MHC-II and costimulatory molecule expression and, in contrast to neutrophils and DC, produce inducible NO synthase. Although neutrophils and monocytes from infected mice produce TNF-α and IL-1β upon ex vivo culture, DC do not. In addition, although recruited monocytes internalize Salmonella in vitro and in vivo they did not induce the proliferation of OT-II CD4+ T cells after coincubation with Salmonella expressing OVA despite their ability to activate OT-II cells when pulsed with the OVA323–339 peptide. We also show that recruited monocytes enter the PP of infected mice independently of the mucosal addressin cell adhesion molecule-1 (MADCAM-1). Finally, recruited but not resident monocytes increase in the blood of orally infected mice, and MHC-II up-regulation, but not TNF-α or iNOS production, occur already in the blood. These studies are the first to describe the accumulation and function of monocyte subsets in the blood and GALT during oral Salmonella infection.


Recruitment of bone marrow-derived phagocytic monocytes is a hallmark of inflammation induced by infection or other means (1, 2). In response to inflammatory stimuli, monocytes are released from the bone marrow into the blood and home to tissues (1–8). The recruitment of monocytes and neutrophils from the blood to infected tissues is a requirement for ensuring host survival to infection (9–13).

Monocytes circulating in the blood are composed of a heterogeneous population of cells that can be divided into distinct phenotypic and functional subsets in humans, rodents, and other species (3, 8, 14, 15). In murine blood, Gr-1intCCR2highCX3CR1low (int, intermediate) monocytes have been described where the latter is more prone to migrate to inflammatory sites while the former migrates to tissues under steady-state conditions (3, 8, 14, 16). It is suggested that Gr-1intCCR2highCX3CR1low monocytes are more immature monocytes newly released from bone marrow that undergo a maturation process and become Gr-1intCCR2highCX3CR1high monocytes (8, 14). In addition to maturation, monocytes, under the influence of local stimuli, have the potential to differentiate into dendritic cells (DC), macrophages, and Langerhans cells (3, 16–25). Thus, recent in vivo studies of monocytes are beginning to elucidate the complex nature of this pivotal mononuclear phagocyte population in steady-state and inflammatory conditions. However, relatively little is known about the role of the recently identified monocyte subsets in bacterial infections, and no information is yet available on the function of these populations in the GALT after oral bacterial challenge.

Salmonella enterica is an intracellular bacterial species that contains several serovars of food and waterborne pathogens, such as the human pathogen serovar Typhi and its cousin serovar Typhimurium (Salmonella enterica serovar Typhimurium). Although S. enterica serovar Typhimurium causes a localized infection of the gastrointestinal tract in humans, it causes a systemic infection resembling typhoid fever in mice. After the oral infection of mice, S. enterica serovar Typhimurium (or Salmonella for brevity) penetrates the intestinal epithelium using M cell-dependent and independent pathways (26). The first organs targeted by orally acquired Salmonella are the Peyer’s patches (PP) and the mesenteric lymph nodes (MLN) (27, 28), the lymphoid organs that drain the intestine and initiate immune responses to gastrointestinal Ags. Although the PP are directly seeded by bacteria that cross the intestinal epithelium, bacteria arrive in the MLN via intestinal lymph, likely transported by DC (26, 29). The bacteria also spread to the spleen and liver via the blood (29). The control of Salmonella early during infection is dependent on IL-12, IFN-γ, and TNF-α, molecules that enhance the microbicidal capacity of phagocytes, and mice lacking any of these cytokines are more susceptible to infection (30). The

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production of reactive nitrogen intermediates via the inducible NO synthase (iNOS) is also important to controlling Salmonella infection (30).

Studies in mice made neutropenic with a depletion strategy using mAb RB6-8C5, which primarily recognizes Ly6G but also cross-reacts with Ly6C (31), have concluded that neutrophils are important for host survival to Salmonella (32, 33). Although this general conclusion is not debated, these studies may also have depleted monocytes, particularly inflammatory monocytes that express Ly6C and are recognized by RB6-8C5 mAb (3, 7, 8, 34). Similarly, studies addressing the role of macrophages during Salmonella infection obtained in studies using chemical depletion methods (35) should be considered with the caveat of monocyte depletion (8) and the possible effects on bystander phagocytes.

Although the importance of mononuclear phagocytes in host survival to Salmonella infection is not disputed, the related phenotypic and functional nature of neutrophils, monocytes, and tissue macrophages (5, 34, 36–39), combined with the recent data revealing monocyte subsets with distinct roles (3, 7, 8), underscores the need for precise analysis of these populations during infection with this intracellular pathogen. This is particularly true for the organs that are the immediate targets of Salmonella after penetration of the intestinal epithelial barrier, the PP, and the MLN.

These issues are addressed here by performing direct ex vivo five- to seven-color flow cytometry analysis of cells accumulating in the PP and MLN during the first few days of oral Salmonella infection of mice. We characterize the influx of monocytes and neutrophils into the gut-draining lymphoid tissues and show that recruited monocytes are the major producers of iNOS and TNF-α, most of which is made by uninfected bystander monocytes. We also examine the capacity of the different myeloid lineage populations recruited to infected PP and MLN to internalize and kill Salmonella in vitro and in vivo, produce TNF-α, IL-1β, and IL-12 upon ex vivo culture, and induce the proliferation of OT-II CD4⁺ T cells after coinoculation with Salmonella expressing OVA or pulsed with OVA peptide. In addition, chemokine receptor expression on the recruited myeloid lineage populations is assessed and the role of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in recruiting monocytes to infected PP is analyzed. Finally, infection-induced recruitment and activation of monocyte subsets in the blood of orally infected mice is examined. These studies are the first to characterize recruited monocytes in PP and MLN after oral Salmonella infection and assess the number and function of their counterparts in the blood of orally infected mice.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories. IFN-γ−/−, IL-12p40−/−, RAG−/− mice and OT-II mice, all backcrossed more than nine generations on the C57BL/6 background, were bred at the Experimental Biomedicine Animal Facility of Göteborg University, Göteborg, Sweden. Mice were used at 8 to 12 wk of age and provided with food and water ad libitum. All experiments were performed using protocols approved by the Swedish government’s animal ethical committee and followed institutional animal use and care guidelines.

Bacterial strains

The S. enterica serovar Typhimurium χ3181, SR11 derivative χ4666 (40) was used for the studies shown in Fig. 1, A and B, and Figs. 3, 5, and 8. S. enterica serovar Typhimurium SL1344 and its enhanced GFP (eGFP)-expressing derivative SMO22 were used where bacterial uptake in vivo was examined (see Fig. 4) (41). Strain 14028r (42) expressing OVA (43) was used for cytokine production and bacterial uptake experiments (Fig. 2, C–E, and Fig. 6B). Strain χ550 expressing OVA-GFP or GFP (44) was used for in vitro infection in the Ag presentation assays (see Fig. 7). Strain χ8554 (SR11 Δaosa16 rpsL hisG) was used for in vivo infection of mice in Fig. 2, C and D, and Figs. 6, 7, and 9. Bacteria were grown in Miller’s Luria-Bertani (LB) broth or Lennox (χ8554) broth overnight at 37°C. For SL1344 and SMO22 the medium was supplemented with antibiotics (SL1344, 100 μg/ml streptomycin; SMO22, 50 μg/ml kanamycin and 100 μg/ml streptomycin). 14028r/OVA was grown on agar supplemented with 50 μg/ml carbenicillin. The bacterial suspension was diluted and the OD was measured at 600 nm. After centrifugation, the bacteria were resuspended at the appropriate concentration in sterile PBS. The actual bacterial dose administered was determined by the serial plating of bacteria on LB agar plates.

Infection of mice

Mice were given 0.1 ml of 1% NaHCO₃ intragastrically 10 min before infection. C57BL/6 or RAG−/− mice were then infected intragastrically with 2–8 × 10⁸ bacteria in 200 μl of PBS. IFN-γ−/− and IL-12−/− mice are more susceptible to Salmonella and were given 5–8 × 10⁸ bacteria in 200 μl PBS intragastrically. Only IFN-γ−/− and IL-12−/− mice with a bacterial load similar to that of wild-type mice were included. In experiments where cell interactions with eGFP-expressing bacteria in vivo were studied (Fig. 4), mice were infected intragastrically with 10⁵ SMO22 or SL1344 bacteria in 200 μl of PBS. In experiments using χ8554 (Fig. 2, C–E, and Figs. 6, 7, and 9), mice were infected intragastrically with 8 × 10⁸ bacteria in 200 μl of PBS. Mice were sacrificed after 2–5 days and the bacterial load in PP, MLN, spleen, and blood was determined by plating serial dilutions of single cell suspensions on LB or Lennox (χ8554) agar plates.

Cell preparation

Mice were sacrificed 2–5 days postinfecion and the spleen, MLN, and PP were removed. Blood was collected from the heart by vascular perfusion with 5 ml of PBS containing 4 mM EDTA. The organs were digested with 0.45 mg/ml Liberase CI (Roche) in HBBS for 30 min at 37°C and pipetted into a single cell suspension or pressed through nylon mesh (100 μm). Splenocytes and blood were treated with 0.14 M NH₄Cl for 5 min at room temperature once or twice, respectively, to lyse RBC, and debris was removed by filtration. Cells were then washed three times with HBSS and resuspended in RPMI 1640 (Invitrogen Life Technologies) with heat-inactivated 10% FBS (PAA Laboratories). The total number of viable cells per organ was determined by trypan blue exclusion. For intracellular cytokine staining, cell suspensions were centrifuged at 500 × g for 3 min, the supernatant was removed, and complete RPMI 1640 supplemented with 10% FBS and incubated at 37°C in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) for 4 h. For bacterial uptake studies in vivo, CD11b-expressing cells were enriched from PP and MLN pooled from 2–5 mice by magnetic separation using autoMACS (Milteny Biotech) according to manufacturer’s protocol.

Flow cytometry

Single cell suspensions were washed in FACS buffer (HBSS containing 3% FBS, 1 mM EDTA, and 10 μM HEPES) that was used throughout the surface staining procedure. FC receptors on cells were blocked by incubating with the anti-FcγRII/III mAb 2.4G2 for 15 min at 4°C. Cells were then stained for 20 min at 4°C with the following mAbs that were biotinylated with a secondary biotin-conjugated Ab for 20 min, washed, and blocked with the primary Ab for 45 min. After washing, cells were incubated with a secondary biotin-conjugated Ab for 20 min, washed, and blocked with opposition of their counterparts in the blood of orally infected mice.
the FcγRII/III mAb 2.4G2 for 15 min. Finally, the cells were incubated with the additional mAbs to stain surface molecules and with streptavidin-allophycocyanin for 20 min.

When intracellular staining was performed, the samples were washed after surface staining and fixed in 2% formaldehyde in PBS for 20 min at room temperature. After the cells were washed in permeabilization buffer (HBSS containing 0.5% saponin and 0.5% BSA (Sigma-Aldrich)), which was subsequently used throughout the staining procedure, they were incubated for 30 min at room temperature with one or more of the following mAbs: anti-TNF-α (clone MP6-XT22), anti-IL-12p40 (clone C15.6), anti-IFN-γ (clone 37895.11) (all purchased from BD Pharmingen), anti-CD68 (clone FA/11; Serotec), *S. enterica* serovar Typhimurium O-4 (clone 1E6; Abcam), and the appropriate isotype control or iNOS Ab (M-19) (Santa Cruz Biotechnology). Staining with rabbit anti-iNOS was followed by incubation with allophycocyanin-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) for 30 min at room temperature. An anti-Salmonella O-4 mAb was conjugated to Pacific Blue according to the manufacturer’s protocol.

Cells or bacteria (strains SMO22 and SL1344) were acquired on an LSR-II flow cytometer (BD Biosciences) using DIVA software (BD Biosciences) and analyzed using FlowJo software (Tree Star). Whereas the absolute number of the cell populations is reported for the MLN and the spleen, the number and size of PP differs in individual mice and thus the percentage rather than the absolute number of cells in a given population was calculated.

**Bacterial survival assay and Ag processing and presentation assays**

Cell suspensions were made from the MLN, spleen, and blood pooled from 10 mice at day 4 postinfection as described above. Cells from the spleen and blood were subsequently pooled (to get enough cells to work with) and depleted of B, T, and NK cells by magnetic separation using autoMACS (Milteny Biotech) after incubation with a mixture of anti-CD19 beads, anti-CD90 (Thy1.2) beads, and anti-NK (DX5) beads according to the manufacturer’s protocol (Milteny Biotech). MLN cells were likewise depleted of T, B, and NK cells. After staining for flow cytometry, cells were sorted into CD11chigh DC, Ly6C<sub>high</sub> monocytes, Ly6C<sub>low</sub> monocytes, and Ly6G<sub>high</sub> neutrophils using a FACSaria flow cytometer (BD Biosciences) and DIVA software (BD Biosciences). The different cell populations were seeded at 1.5 × 10<sup>5</sup> cells/well in round-bottom 96-well plates in complete medium (RPMI 1640 Glutamax-1 containing 10% FBS (PAA Labortories), 2 mM MEM sodium pyruvate, 20 mM HEPES, 0.05 mM 2-ME, and 2.5 µg/ml fungizone (all from Invitrogen Life Technologies)). For bacterial survival assays, sorted Ly6G<sub>high</sub> neutrophils and Ly6C<sub>high</sub> monocytes were infected with strain 14028r, centrifuged at 270 g for 4 min, and incubated for 2 h at 37°C. The bacteria-to-cell ratio was between 5:1 and 18:1. After washing three times in complete medium containing 80 µg/ml gentamicin, the cells were resuspended in the medium and incubated at 37°C for an additional 1 or 18 h. Cells were lysed with 0.2% Triton X-100 with vigorous pipetting and incubated at room temperature for 10 min. The supernatant was then serially diluted and plated onto LB agar plates. Bacterial colonies were counted after overnight incubation at 37°C and the total number of bacteria recovered was calculated. As controls for determining the number of bacteria not internalized by the cells but viable in the supernatant, cells were preincubated with 20 µg/ml CDD (cytochalasin D) 1 h before the addition of bacteria and present for the initial 2 h of bacterial infection.

For Ag-presenting assays, titrated numbers of χ4550-OVA-GFP or χ4550-GFP (no OVA) were added to the cells. Alternatively, the OVA<sub>323–339</sub> peptide was added at a concentration of 0.1, 1, or 10 µg/ml. Cells were then centrifuged for 270 × g for 4 min and incubated at 37°C. After 2 h,
The gut-draining lymphoid tissues PP and MLN are the first organs to accumulate phagocytic cells after oral infection (27). However, little is known about the changes in the number, phenotype, and function of phagocytic cell populations, particularly the recently described monocyte subsets, responding to this oral pathogen in these organs. To address these issues, mice were orally infected with Salmonella (Fig. 1), increased dramatically in the MLN and PP of infected mice starting at day 3 postinfection (Fig. 1), and at 36 h for IL-1\(\beta\) and was measured by ELISA. Results are expressed as mean \(\pm\) SEM (pg/ml) of 1–3 independent experiments with duplicate samples. MACS-purified CD11b\(^+\) cells from naive mice are shown as a naive control. ND, not detected.

**Adaptive transfer and blocking experiments**

Donor monocytes and neutrophils were negatively enriched using autoMACS and anti-CD49b (clone DX5) beads (Miltenyi Biotech) from pooled splenocytes and the blood of 10–15 RAG\(^{-/-}\) mice infected 3 days earlier with Salmonella \(\chi_{4666}\). Donor T cells were negatively enriched from the pooled spleen and MLN of naive C57BL/6 mice using autoMACS and a CD4 isolation kit (Miltenyi Biotech). The donor monocytes, neutrophils, and T cells were then pooled and CFSE labeled as described above. Cells (10–15 \times 10^5) containing ~35% T cells and ~39% CD11b\(^+\)NK1.1\(^+\) cells were injected i.v. into C57BL/6 mice that were infected 3 days earlier with \(\chi_{4666}\). Some of the recipient mice were given 150 \(\mu\)g of the anti-MAdCAM-1 mAb MECA-367 (45) (BD Pharmingen) i.v. 6–8 h before cell transfer. Control animals received either 150 \(\mu\)g of the isotype control mAb 9B5 (anti-human CD44), kindly provided by A. Hänninen (University of Turku, Turku, Finland) or nothing. Twelve to 16 h after adoptive transfer, PP, MLN, spleen, and blood were harvested from recipient mice, and stained for cell numbers by flow cytometry as described above.

**Results**

**Phagocytic cells accumulate in the GALT after oral infection**

The gut-draining lymphoid tissues PP and MLN are the first organs seeded by Salmonella after oral infection (27). However, little is known about the changes in the number, phenotype, and function of phagocytic cell populations, particularly the recently described monocyte subsets, responding to this oral pathogen in these organs. To address these issues, mice were orally infected with Salmonella and cells from PP and MLN were analyzed by five- to seven-color flow cytometry early during infection. Populations with differential expression of CD68 and Gr-1 responding to oral infection were apparent (Fig. 1). In particular, two populations that were relatively rare in naive mice, CD68\(^{hi}\)Gr-1\(^{hi}\) (where int stands for intermediate) and CD68\(^{hi}\)Gr-1\(^{int}\) cells (gate R1 and R2, respectively, in Fig. 1), increased dramatically in the MLN and PP of infected mice starting at day 3 postinfection (Fig. 1, A and C and D). Although the increase in both populations was large, CD68\(^{hi}\)Gr-1\(^{int}\) (R2) cells remained more abundant than gated R1 cells and comprised 1–3% of total cells per organ at day 5 postinfection.
To further characterize the two populations responding to oral *Salmonella* infection, the expression of a number of surface and costimulatory molecules was examined (Fig. 1E). The gated CD68<sup>+</sup>Gr-1<sup>+</sup> (R1) cells in infected mice lacked F4/80 and CD11c expression but had a very high level of CD11b. Few cells within this gate were positive for MHC-II, CD80, or CD86. Both myeloid and lymphoid DC are included in this gate. Although most cells within the R2 gate of infected MLN were CD11c<sup>+</sup> and CD11b<sup>+</sup>, they had several features that distinguished them from R1 cells. This included the expression of F4/80 and a significant level of MHC-II by the majority of the gated cells. R2 cells from infected mice had increased CD80 and CD86 expression compared with naive mice, a fraction of the cells were CD62L<sup>+</sup>, and some expressed a low level of CD11c (Fig. 1E). Thus, infection-induced R2 cells are phenotypically distinct from R1 cells.

Although the very low level of CD11c on gated R2 cells suggested that they were not conventional DC, which in the mouse are characterized by high surface expression of CD11c, MHC-II, and costimulatory molecules (46), the phenotype of gated R2 cells was consistent with previous data (46–48). Although most of the gated R2 cells were positive for CD62L, this included the expression of F4/80 and a significant level of MHC-II by the majority of the gated cells. R2 cells from infected mice had increased CD80 and CD86 expression compared with naive mice, a fraction of the cells were CD62L<sup>+</sup>, and some expressed a low level of CD11c (Fig. 1E).

The mono/mac that accumulate in PP and MLN are the main producers of TNF-α and iNOS during infection

To assess the function of the myeloid cells recruited to PP and MLN during oral *Salmonella* infection, their capacity to produce TNF-α and iNOS, effector molecules important in controlling *Salmonella* (30), were assessed by direct ex vivo double intracellular staining. Mono/mac producing TNF-α alone, iNOS alone, or both simultaneously were induced by *Salmonella* infection (Fig. 2A and Table I). Mono/mac were the predominant producers of these anti-bacterial molecules, accounting for 50–70% of all TNF-α<sup>+</sup> and 80–90% of all iNOS<sup>+</sup> cells in MLN. Moreover, 20–25% of the mono/mac that stain positive for iNOS were also positive for TNF-α.

In contrast to mono/mac, very few neutrophils or DC stained positive for iNOS (Fig. 2B and Table I). Similar results were also found in the PP and spleen of orally infected mice (Table I). Some increase in cells staining positive for intracellular TNF-α was apparent among neutrophils and DC in infected mice (Table I).

<table>
<thead>
<tr>
<th>Cell population</th>
<th>PP</th>
<th>MLN</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>iNOS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TNF-α&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DP&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neutrophils (R1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>0.4 ± 0.9</td>
<td>10.2 ± 7.6</td>
<td>#</td>
</tr>
<tr>
<td>Infected</td>
<td>2.6 ± 3.4**†</td>
<td>10.8 ± 7.7**</td>
<td>#</td>
</tr>
<tr>
<td>Mono/mac (R2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>1.3 ± 1.6</td>
<td>7.9 ± 5.3</td>
<td>#</td>
</tr>
<tr>
<td>Infected</td>
<td>31.6 ± 13.42</td>
<td>28.9 ± 10.6‡</td>
<td>8.9 ± 2.9</td>
</tr>
<tr>
<td>DC (R3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>0.8 ± 1.3</td>
<td>0.8 ± 0.4</td>
<td>#</td>
</tr>
<tr>
<td>Infected</td>
<td>10.6 ± 6.2**‡</td>
<td>1.4 ± 0.5*#</td>
<td>#</td>
</tr>
</tbody>
</table>

The data in Fig. 1 show that the predominant cellular change that occurs in the GALT after oral *Salmonella* infection is the rapid accumulation of cells with a phenotype distinct from neutrophils and DC. Moreover, the phenotype of R2 cells suggests they may be monocytes recruited from the blood, as has been shown in peritoneal or i.v. inflammation models and in lymph nodes draining inflamed skin (1–4, 6–8, 37). Because monocyte refers to a population circulating in the blood and macrophage indicates a tissue resident differentiated cell, we refer to the cells that accumulate in infected PP and MLN as monocyte/macrophage (mono/mac).

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Table I. Production of iNOS and TNF-α in phagocytic cells of naïve or *Salmonella*-infected mice at day 4 post infection

<table>
<thead>
<tr>
<th>Cell population</th>
<th>PP</th>
<th>MLN</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
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<td>0.4 ± 0.9</td>
<td>10.2 ± 7.6</td>
<td>#</td>
</tr>
<tr>
<td>Infected</td>
<td>2.6 ± 3.4**†</td>
<td>10.8 ± 7.7**</td>
<td>#</td>
</tr>
<tr>
<td>Mono/mac (R2)</td>
<td></td>
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<tr>
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<tr>
<td>DC (R3)</td>
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<td>#</td>
</tr>
<tr>
<td>Infected</td>
<td>10.6 ± 6.2**‡</td>
<td>1.4 ± 0.5*#</td>
<td>#</td>
</tr>
</tbody>
</table>

<sup>a</sup> The mean percentage of positive cells for either iNOS or TNF-α within the respective cell population ± SD of groups of 2–17 mice from 2–4 independent experiments is shown.

<sup>b</sup> DP, Cells double positive for both TNF-α and iNOS.

<sup>c</sup> ND, Not done.

# Too few cells (<50) to accurately determine; * and **, p < 0.05 and p < 0.005, respectively, when compared with infected mono/mac. † and ‡, respectively, with the same population of cells from naive mice. All p values were calculated using the Mann-Whitney test.
resulted in enhanced IL-1β production (Fig. 2, D and E). IL-12p70 was not detected in the supernatant of any of the sorted cell types cultured ex vivo with or without Salmonella (data not shown). Likewise, an increase in monocytes from the MLN or spleen of mice infected 3 or 5 days earlier with Salmonella that stained positive for intracellular IL-12p40 above the level detected in naïve mice was not detected. Despite the fact that IL-12p70/IL-12p40 has a role during Salmonella infection (30), the cellular source(s) of this cytokine remain elusive.

Thus, mono/mac, defined as CD68<sup>hi</sup>Gr-1<sup>lo</sup> cells, express MHC-II and costimulatory molecules (Fig. 1E) and are the main sources of TNF-α and iNOS early during Salmonella infection. Moreover, the majority of mono/mac in infected tissues produce TNF-α or iNOS rather than both simultaneously.

**Impaired expression of iNOS and MHC-II but not TNF-α in infected IFN-γ<sup>-/-</sup> and IL-12p40<sup>−/−</sup> mice**

We next asked whether iNOS and TNF-α produced by recruited mono/mac early during infection depended on IFN-γ and IL-12. First, the accumulation of mono/mac and neutrophils in the PP, MLN, and spleen of infected IFN-γ<sup>-/-</sup> and IL-12p40<sup>−/−</sup> mice with a bacterial burden similar to that of infected wild-type mice 4 days postinfection was not compromised (data not shown). Likewise, the frequency of mono/mac producing TNF-α in PP, MLN, and spleen was the same in wild-type, IFN-γ<sup>-/-</sup>, and IL-12p40<sup>−/−</sup> mice with a similar bacterial burden (Fig. 3, A–C). In contrast, iNOS production by mono/mac in all three organs in infected IFN-γ<sup>-/-</sup> and IL-12p40<sup>−/−</sup> mice was nearly absent (Fig. 3, A and B).

We also wished to determine whether the infection-induced increase in MHC-II in the mono/mac of infected wild-type mice was IFN-γ-dependent. Indeed, Salmonella-induced MHC-II up-regulation on mono/mac was severely compromised in IFN-γ<sup>-/-</sup> and IL-12p40<sup>−/−</sup> mice (Fig. 3D). This is in contrast to MHC-II expression on DC, which was similar and high on CD11c<sup>hi</sup> (R3) cells in infected wild-type and knockout mice. Similar results were seen in the spleen (data not shown). We next examined the cellular source(s) of IFN-γ in the PP and MLN responsible for infection-induced mono/mac activation. These data revealed that NK and NKT cells (NK1.1<sup>+</sup> TCRα<sup>+</sup>) and some T cells (TCRαβ<sup>+</sup> NK1.1<sup>+</sup>) are the main producers of IFN-γ 4 days postinfection (Fig. 3E). Thus, the induction of iNOS and MHC-II expression but not TNF-α production by mono/mac during oral Salmonella infection is dependent on IL-12 and IFN-γ, and NK/NKT cells and T cells are the predominant sources of IFN-γ in PP and MLN early during infection.

**Uptake of eGFP-expressing Salmonella by phagocytic cells**

To examine the relative uptake of bacteria by mono/mac and neutrophils in the same individual, mice were orally infected with Salmonella expressing eGFP and bacteria-associated cells were identified by flow cytometry. To get enough events to accurately assess the phenotype of eGFP<sup>+</sup> cells, CD11b<sup>+</sup> cells were first enriched by positive selection using MACS. In addition, an analysis of eGFP fluorescence negated using FITC-conjugated anti-CD68 (Fig. 1A), so an alternate strategy using CD11b, Gr-1, and F4/80 was used to identify the phagocytic cells (Fig. 4A) (7). To conclude that this strategy allowed identification of the neutrophil

**FIGURE 3.** Defective iNOS and MHC-II but not TNF-α production in mono/mac from infected IFN-γ<sup>-/-</sup> and IL-12p40<sup>−/−</sup> mice. Wild-type (WT), IFN-γ<sup>-/-</sup>, and IL-12p40<sup>−/−</sup> mice were orally infected with Salmonella and 4 days (PP and MLN) or 5 days (spleen) later cells were stained with 7-AAD, anti-CD68, anti-Gr-1, anti-CD11c, and either anti-TNF-α and anti-iNOS (A and B) or anti-MHC-II (D) and analyzed by six- (A and B) or five-color (D) flow cytometry. Mono/mac and DC were gated as in Fig. 1A. A, The dot plots show intracellular staining for TNF-α and iNOS on cells from the MLN of wild-type, IFN-γ<sup>-/-</sup>, and IL-12p40<sup>−/−</sup> mice with a similar bacterial burden (shown in C). The numbers represent the percent positive cells in the quadrant. B, TNF-α (left) and iNOS (right) production by mono/mac in the PP, MLN, and spleen of infected wild-type, IFN-γ<sup>-/-</sup>, and IL-12p40<sup>−/−</sup> mice with a similar bacterial burden (shown in C). TNF-α and iNOS production in naïve mice is shown in Table 1. Error bars indicate ± SD. The p values (*, p < 0.01; **, p < 0.001) are from the Mann-Whitney U test for infected C57BL/6 mice compared with infected IFN-γ<sup>-/-</sup> or IL-12p40<sup>−/−</sup> mice. C, Bacterial recovery from PP, MLN, and spleen 4 days postinfection. D, MHC-II expression by mono/mac in PP and MLN (two left histograms) and DC in MLN (right histogram) in wild-type, IFN-γ<sup>-/-</sup>, and IL-12p40<sup>−/−</sup> mice with a similar bacterial burden (shown in C) at day 4 postinfection. E, IFN-γ production by NK1.1<sup>+</sup> cells (NK and NKT cells) and T cells (TCRαβ<sup>+</sup> NK1.1<sup>+</sup>) gated as in the upper dot plot in PP and MLN at day 4 postinfection (upper panel) is shown. Staining for IFN-γ in IFN-γ<sup>-/-</sup> mice is used as a control (lower row). A–E Data were obtained in at least three independent experiments and each group contains 4–16 mice.
FIGURE 4. Bacterial uptake in vivo by phagocytic cells in MLN and PP at day 4 postinfection. Mice were orally infected with Salmonella expressing eGFP or the parental strain not expressing eGFP and after 4 days CD11b⁺ cells were purified by MACS from MLN and PP. MACS-enriched cells were then stained with anti-CD11b, anti-Gr-1, and 7-AAD and anti-F4/80, anti-MHC-II, anti-TNF-α, or the appropriate control isotype and analyzed by five-color flow cytometry. A, CD11b-enriched cells were gated into CD11b⁺Gr-1⁺ cells and further gated into F4/80high (F2) or F4/80low (F1) populations. B, These two populations were then backgated into a CD68 vs Gr-1 plot to confirm that CD11b⁺Gr-1⁻ F4/80high cells correspond to CD68⁺Gr-1⁺ (R1 cells) and that CD11b⁺Gr-1⁺ F4/80low cells correspond to CD68⁺Gr-1⁻ (R2 cells). C, The percentage of eGFP⁺ cells within gated neutrophils (F1) and monocytes (F2) in PP (left) and MLN (right) of a Salmonella-infected mouse 4 days postinfection. The upper row is from a wild-type mouse infected with Salmonella expressing eGFP (Sal eGFP) while the middle row is a wild-type mouse infected with the parental Salmonella strain not expressing eGFP (Sal). The lower row of dot plots is from an IFN-γ⁻/⁻ mouse infected with Salmonella expressing eGFP. Data are representative of five independent experiments. The Mann-Whitney U test comparing C57BL/6 or IFN-γ⁻/⁻ cells infected with Salmonella expressing eGFP to mice infected with Salmonella not expressing eGFP was used for statistical analysis. The value of p = 0.05 was obtained for all groups. Samples contained 34–2550 eGFP⁺ events. D, CD11b⁺Gr-1⁺ cells from the MLN and PP of mice infected 4 days earlier and mono/mac cells studied above, CD11b⁺Gr-1high F4/80⁻ and CD11b⁺Gr-1low F4/80⁺ were backgated into a CD68 vs Gr-1 plot (Fig. 4B). This showed that the F1 and F2 populations defined by CD11b⁺Gr-1⁻, and F4/80 identified the majority (63–95% in three independent experiments) of mono/mac (R1) and neutrophils (R2), respectively, with little cross-contamination between the two populations (Fig. 4, A and B) (7).

The results in Fig. 4C show a slightly higher fraction of eGFP⁺ neutrophils (F1 cells) compared with mono/mac (F2) (~1.6-fold difference) in the PP and MLN of infected wild-type mice. In infected IFN-γ⁻/⁻ mice, very similar fractions of neutrophils and mono/mac were eGFP⁺ in both PP and MLN (1.2-fold difference). The somewhat higher fraction of eGFP⁺ cells in the MLN of infected IFN-γ⁻/⁻ mice compared with wild-type mice reflects the higher number of bacteria recovered from IFN-γ⁻/⁻ mice in these experiments (data not shown) due to the compromised capacity to kill Salmonella in the absence of IFN-γ (30).

To investigate the accuracy of detecting cell-associated bacteria in wild-type and IFN-γ⁻/⁻ mice using eGFP fluorescence as the readout, two types of experiments were performed. First, the stability of eGFP in CD11b⁺ cells from infected wild-type and IFN-γ⁻/⁻ mice was assessed. These data showed similar eGFP stability, as 94 ± 6.3% (n = 7) and 94 ± 2.2% (n = 6) of bacterial colonies recovered from CD11b⁺ cells isolated from infected wild-type and IFN-γ⁻/⁻ mice, respectively, were eGFP⁺. Second, the detection bacteria by eGFP fluorescence was compared with the use of an anti-Salmonella O-4 LPS Ab. These studies revealed that 1.3–4.9% of CD11b⁺ cells from PP and MLN pooled from infected wild-type mice stained positive for O-4 reactivity, while eGFP fluorescence was detected in 0.4–2% of the cells in the same infected individuals. Identical percentages were obtained for infected IFN-γ⁻/⁻ mice. Although the down-regulation of eGFP fluorescence in vivo cannot be eliminated as a contributor to this difference, the greater reactivity of an anti-LPS Ab is not unexpected because it will recognize LPS on intact bacteria as well as bacterial degradation products and shed LPS. In contrast, the detection of bacteria using eGFP fluorescence relies on intact protein emitting fluorescence, a method that will not detect bacteria degraded to a point that negates eGFP fluorescence.

Very few eGFP⁺ events were found among TCRαβ⁺ or B220⁺ cells in infected tissues of the same animals, demonstrating that >80% of all bacteria-associated cells were either neutrophils or mono/mac (data not shown). The relationship between bacterial uptake and effector molecule production was also assessed directly ex vivo (Fig. 4D). Approximately 30–40% of eGFP⁺ CD11b⁺Gr-1⁻ F2 cells were also iNOS⁺ (data not shown), demonstrating the tendency of bacteria-associated cells to produce this effector molecule. However, while ~30–40% of all CD11b⁺Gr-1⁻ F2 cells expressed iNOS (Fig. 3B), only a minor fraction of iNOS⁺ or TNF-α⁺ CD11b⁺Gr-1⁻ cells were eGFP⁺ (Fig. 4D). This suggests that most iNOS and TNF-α⁺-producing cells are bystander cells, as has been observed with other pathogens (5, 7, 12). In
addition, bacterial uptake did not appear to influence the infection-induced up-regulation of MHC-II on mono/mac either in wild-type or IFN-γ−/− hosts (see Fig. 4E for PP, MLN data not shown). Similarly, bacterial uptake did not restore the ability of mono/mac from infected IFN-γ−/− mice to produce iNOS (Fig. 3A and data not shown).

Accumulation of cells in the blood of orally infected mice

Having established that neutrophils and cells with a phenotype suggesting a relationship to monocytes (3, 9, 13, 34, 50) (our so-called mono/mac population) accumulated and exerted effector functions in the gut-associated lymphoid tissues of orally infected mice, we asked when these cells appeared in the blood during infection and whether they were activated in the circulation. To this end, the populations defined in PP and MLN based on CD68 and Gr-1 expression were characterized in the blood of naive and Salmonella-infected mice. CD68 vs Gr-1 staining on blood cells revealed abundant R1 and R2 populations similar to those found in the tissues (Figs. 5A and 1A). In naive mice, R1 and R2 were 8 and 4%, respectively, of all leukocytes in blood. Both R1 and R2 populations accumulated in the blood as the infection progressed, increasing 2-fold and 4-fold, respectively, at day 3 postinfection and 2.5-fold and 4.5-fold, respectively, at day 5 (Fig. 5, A and C).

To further characterize the cell populations in blood during infection and compare them with the ones found in infected tissues, the expression of myeloid markers and costimulatory molecules on the blood populations was analyzed (Fig. 5D). The phenotype of gated Gr-1highCD68int (R1) cells in the blood was very homogeneous and similar in both infected and naive mice (Fig. 5D). Essentially, all blood R1 cells expressed CD11b and CD62L but lacked F4/80, CD11c, MHC-II, CCR2, and the costimulatory molecules CD80 and CD86. In addition, gated R1 cells (as well as R2 and R4 cells) were negative for CD19, TCR, and NK1.1 (data not shown).

Like their tissue counterparts, blood CD68highGr-1int (R2) cells from infected mice were positive for CD11b, F4/80, and CCR2 (Fig. 5D and data not shown). The adhesion molecule CD62L was expressed on some blood R2 cells in naive mice, and the fraction of CD62L+ cells increased in the blood of Salmonella-infected mice. Similar to R2 cells in the MLN of infected mice, R2 cells in the blood expressed much higher MHC-II in infected compared with naive animals. In contrast, however, the up-regulation of CD86 and particularly CD80 on R2 cells in the blood was modest compared with that seen in infected MLN (Figs. 1E and 5D).

Cells consistent with the phenotype of murine DC present in the tissues of naive and infected mice (CD11cintMHC-II−) were not found in the blood (Fig. 5B and data not shown) (51). However, two populations of monocytes, Gr-1−CD62L−CCR2− inflammatory and Gr-1−CD62L−CCR2+ resident monocytes, have been described in mouse blood (3). The Gr-1−CD62L−CCR2+ phenotype of gated R2 cells is consistent with the phenotype of inflammatory monocytes, while Gr-1−CD62L+ cells in R4 share features with resident monocytes (Fig. 5, A and D) (3). R2 cells increased in the blood of infected mice while R4 cells did not (Fig. 5C). Moreover, most of the cells within the R4 gate, putative resident monocytes, express an intermediate level of CD11c and up-regulate MHC-II during infection. This suggests a possible relationship to, or capacity to become, DC. Cells within gate R4 appear to be the best candidate for resident monocytes, as the CD11cint cells in gate R5 in Fig. 5B were mostly (>80%) NK1.1+CD11bint NK cells (data not shown), consistent with other...
reports (51, 52). The remaining NK1.1− cells in R5 were CD68highGr-1lowCD11bint and fall within gate R4 in Fig. 5A.

Given the efficient activation of mono/mac to produce large amounts of iNOS and TNF-α and to up-regulate MHC-II in the PP, MLN, and spleen during infection, we asked whether this activation already occurs in the blood of infected mice. As seen in Fig. 5D, MHC-II was readily up-regulated on R2 cells, putative inflammatory monocytes in blood. However, no Salmonella-induced iNOS and little TNF-α, 5D, MHC-II was readily up-regulated on R2 cells, putative inflammatory monocytes in blood. However, no Salmonella-induced iNOS and little TNF-α, above the level seen in naive mice were detected in this population (Fig. 5E). Together, the data in Fig. 5 show that two subsets of monocytes resembling resident (R4) and inflammatory monocytes (R2) can be identified in the blood of Salmonella-infected mice. R2 but not R4 monocytes increase in the blood during Salmonella infection and both have increased MHC-II expression but do not produce anti-bacterial effector molecules. Moreover, neutrophils increase in the blood of Salmonella-infected mice, while cells with the features of tissue DC are not readily apparent in the blood of naive or infected animals.

Bacterial uptake and Ag presentation capacity

To examine the phagocytic activity and killing capacity of monocytes and neutrophils, these cells were isolated from blood and spleen at day 4 postinfection, pooled, and depleted of T, B, and NK cells by MACS. The remaining cells were sorted into Ly6Chigh monocytes (CD11b+Ly6GlowLy6Cint) and neutrophils (CD11b+Ly6GhighLy6Cint) (Fig. 5A). Because CD68 is expressed intracellularly and requires a staining procedure using fixation and permeabilization that kills the cells, we changed the gating strategy and used CD11b, Ly6C, and Ly6G to identify mono/mac and neutrophils (7, 8, 31, 34, 37, 53). Anti-TCR, CD19, NK1.1, and CD11c were also included in the staining to assure that no T cells, B cells, NK cells, or DC were included in the gates. To conclude that this strategy allowed identification of the mono/mac and neutrophils previously defined by CD68 and Gr−1, CD11b+Ly6GlowLy6Chigh monocytes and CD11b+Ly6GhighLy6Cint neutrophils were back gated into a CD68 vs Ly6G plot (Fig. 5A). This showed that 79% of the CD11b+Ly6GlowLy6Chigh cells fell within the mono/mac (R2) gate and 97% of the CD11b+Ly6GhighLy6Cint cells fell into the neutrophil (R1) gate, with little cross-contamination between the two populations (Fig. 5A).

The sorted monocytes and neutrophils were pulsed with Salmonella for 2 h, washed extensively, cultured for an additional 1 or 18 h in medium containing gentamicin, and the intracellular survival of bacteria was examined. After 3 h, both monocytes and neutrophils had phagocytosed bacteria but the neutrophils were more effective (Fig. 6B), which is consistent with the in vivo data (Fig. 4C). After 20 h, few viable bacteria were recovered from the two populations, showing that most of the phagocyotosed bacteria had been killed. The use of CCD in parallel wells showed that the majority of bacteria were actively internalized rather than being attached to the cell surface (Fig. 6B).

Having established that Ly6Chigh monocytes were able to phagocytose bacteria in vitro, we wanted to examine whether they or Ly6Clow monocytes from infected mice were able to induce the proliferation of OT-Il CD4+ T cells. To examine this, Ly6Chigh monocytes, Ly6Clow monocytes, and DC from infected mice were sorted based on expression of CD11b, Ly6C, Ly6G, and CD11c (Fig. 7A), pulsed ex vivo with Salmonella expressing OVA, and subsequently cocultured with CFSE-labeled OT-II CD4+ T cells. DC but not Ly6Chigh or Ly6Clow monocytes induced proliferation of OT-II cells (Fig. 7B). Bacterial titration showed that the T cell response peaked when DC were stimulated using a 5 to 1 bacteria to cell ratio, and that Ly6Chigh monocytes were unable to induce a T cell response at all bacteria to cell ratios tested (Fig. 7C). Furthermore, DC titration with a fixed bacteria to cell ratio (5 to 1) showed that proliferation of OT-II cells decreased as DC number declined (Fig. 7D). The observed proliferation of DC was epitope-specific, because a lack of proliferation was observed when the cells were pulsed with Salmonella not expressing OVA (Fig. 7D). Finally, pulsing with the OVA323-339 peptide showed that Ly6Chigh monocytes induced a modest proliferation of OT-II cells, while both Ly6Clow monocytes and DC efficiently induced proliferation (Fig. 7B).

Together, these results show that Ly6Chigh monocytes and neutrophils phagocytose and kill Salmonella in vitro. Moreover, and in contrast to DC, neither Ly6Chigh or Ly6Clow monocytes induce OT-II T cell proliferation after pulsing with Salmonella expressing OVA although Ly6Clow monocytes induce OT-II proliferation after pulsing with OVA peptide.

Monocytes and neutrophils are recruited to PP despite blocking of MAdCAM-1

Monocytes and neutrophils in blood express the selectin CD62L (Fig. 5D) and the α4 integrin (37, 54, 55). CD62L and α4 partners with β2, in particular, are ligands for MAdCAM-1. MAdCAM-1 is expressed on the high endothelial venules of PP and is critical for lymphocyte homing to this organ (45, 56, 57). To investigate whether MAdCAM-1 is also involved in monocyte and neutrophil recruitment to PP during Salmonella infection, blocking studies were performed. Although T cell recruitment to the PP of Salmonella-infected mice was abrogated in animals treated with the
cell ratio. Alternatively, the cells were pulsed with OVA for 2 h and processed as in Fig. 8. Half of the recipient Salmonella-infected mice were given anti-MAdCAM-1 mAb (MECA367) i.v. 6–8 h before cell transfer. Twelve to 16 h later, PP, MLN, and blood were removed and stained with anti-CD11b, anti-TCRβ, anti-CD19, anti-NK1.1, anti-Ly6C, and anti-Ly6G and analyzed by flow cytometry. A, TCRβ+CD11b+ cells were identified as T cells (left) and CD11b+TCRβ+CD19−NK1.1− cells were further gated into monocytes (Ly6CintLy6Ghigh) or neutrophils (Ly6CintLy6Ghigh) (right) in PP as indicated. B and C. Recruitment of adoptively transferred T cells, monocytes, and neutrophils to PP on day 4 postinfection after blocking i.v with anti-MAdCAM-1 (MECA 367) (upper row) or not blocking (lower row) is shown. The percentage of CFSE+ cells within the population is indicated in the plots. Mice given an isotype control Ab (9B5; anti-human CD44) gave similar results as nontreated mice (not shown). Results are from five independent experiments with a total of 8–10 mice per group. Error bars are the SEM. The p value: (*, p < 0.001) are from the Mann-Whitney U test for anti-MAdCAM-1-treated mice compared with unblocked or isotype-blocked mice.

FIGURE 8. Recruitment of adoptively transferred Ly6Cint monocytes and neutrophils to PP during oral Salmonella infection occurs despite blocking MAdCAM-1. A total of 10–15 × 10^6 CFSE-labeled monocytes, neutrophils, and T cells were injected i.v. into C57BL/6 mice orally infected 3 days earlier with S. Typhimurium. Half of the recipient Salmonella-infected mice were given anti-MAdCAM-1 mAb (MECA367) i.v. 6–8 h before cell transfer. Twelve to 16 h later, PP, MLN, and blood were removed and stained with anti-CD11b, anti-TCRβ, anti-CD19, anti-NK1.1, anti-Ly6C, and anti-Ly6G and analyzed by flow cytometry. A, TCRβ+CD11b+ cells were identified as T cells (left) and CD11b+TCRβ+CD19−NK1.1− cells were further gated into monocytes (Ly6CintLy6Ghigh) or neutrophils (Ly6CintLy6Ghigh) (right) in PP as indicated. B and C. Recruitment of adoptively transferred T cells, monocytes, and neutrophils to PP on day 4 postinfection after blocking i.v with anti-MAdCAM-1 (MECA 367) (upper row) or not blocking (lower row) is shown. The percentage of CFSE+ cells within the population is indicated in the plots. Mice given an isotype control Ab (9B5; anti-human CD44) gave similar results as nontreated mice (not shown). Results are from five independent experiments with a total of 8–10 mice per group. Error bars are the SEM. The p value: (*, p < 0.001) are from the Mann-Whitney U test for anti-MAdCAM-1-treated mice compared with unblocked or isotype-blocked mice.

Chemokine receptor expression on Ly6Cint monocytes, Ly6Cint monocytes, neutrophils, and DC in blood, PP, and MLN of Salmonella-infected mice

To gain further insight into entry of myeloid cells into the gut lymphoid tissues during oral Salmonella infection, we next studied chemokine receptor expression because this receptor/ligand interactions provide important information that direct cell entry into lymphoid tissues (58). Thus, CXCR2, CXCR3, and CCR6 expression on myeloid cells from blood, PP and MLN were analyzed by flow cytometry at day 4 postinfection. Almost all blood neutrophils express CXCR2, while <20% of neutrophils in PP and MLN express this receptor (Fig. 9). In contrast, few if any Ly6Cint monocytes in any of the immune compartments examined were CXCR2 +. The fraction of cells positive for CXCR3 was relatively low for all cell types in all tissues examined, except for neutrophils in MLN where 13% were positive. Finally, CCR6 expression was only found on DC in PP and MLN. Thus, the high expression of CCR2 on Ly6Cint monocytes in blood (R2 cells; Fig. 5D) is consistent with the role of this receptor and its ligand MCP-1 (CCL2) in the release of these inflammatory monocytes into the blood for subsequent entry into infected tissues, as described previously (3, 7, 12, 59). Moreover, the data suggest a potential role for CXCR2 in neutrophil migration and CCR6 in the migration of DC.

FIGURE 7. DC but not Ly6Cint monocytes (Mo hi) or Ly6Cint monocytes (Mo lo) process and present a Salmonella Ag on MHC class II. Cells from the blood and spleen of mice infected 4 days earlier with S. Typhimurium were pooled, depleted of B, T, and NK cells, and stained with anti-CD11b, anti-CD11c, anti-Ly6C, and anti-Ly6G and a mixture of CD19, NK1.1, and TCRβ. A, Cells were sorted into Ly6Cint monocytes, Ly6Cint monocytes, and CD11c+DC as shown. B, A total of 1.5 × 10^5 cells/well were pulsed for 2 h with 1.6% proliferation of the OT-II cells. The fraction of cells positive for CXCR3 was relatively high for all cell types in all tissues examined, except for neutrophils in MLN where 13% were positive. Finally, CCR6 expression was only found on DC in PP and MLN. Thus, the high expression of CCR2 on Ly6Cint monocytes in blood (R2 cells; Fig. 5D) is consistent with the role of this receptor and its ligand MCP-1 (CCL2) in the release of these inflammatory monocytes into the blood for subsequent entry into infected tissues, as described previously (3, 7, 12, 59). Moreover, the data suggest a potential role for CXCR2 in neutrophil migration and CCR6 in the migration of DC.

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that a relatively small fraction of monocytes producing iNOS or these cells observed with other pathogens (60, 61). Some cytokines is also consistent with the complex activities of neutrophils from infected organs and was unaffected in the absence of IL-12 or IL-12- and IFN-γ-dependent fashion. In contrast, MHC-II expression on DC (40, 46, 47). Furthermore, MHC-II expression on Gr-1 high monocytes was greatly increased during infection in an IFN-γ-dependent fashion. In contrast, MHC-II expression on DC (40, 46, 47). Furthermore, MHC-II expression on Gr-1 high monocytes was greatly increased during infection in an IFN-γ-dependent fashion. In contrast, MHC-II expression on DC (40, 46, 47).

The origin of monocytes, neutrophils, and DC from a common myeloid progenitor (36, 39), their overlapping expression pattern of surface molecules (5, 34, 37, 38), and the differentiation capacity of monocytes (3, 17–25) make characterizing tissue-infiltrating myeloid populations complex. In this study we characterized the rapid recruitment of CD68 high Gr-1 high and CD68 high Gr-1 high cells to PP and MLN in the first few days after oral Salmonella infection. The phenotype of the recruited cells was consistent with their identification as neutrophils (CD68 int Gr-1 high) and inflammatory monocytes (CD68 high Gr-1 high) (3, 5, 8, 34, 37, 38). Recruited monocytes were numerically dominant over neutrophils in infected tissues and were major producers of the effector molecules important in controlling Salmonella infection, iNOS and TNF-α.

CD68 high Gr-1 high monocytes recruited to PP and MLN during infection had increased MHC-II, CD80, and CD86 but yet had phenotypic and functional features distinct from DC in the same tissue. For example, low CD11c expression was detected on a small fraction of recruited monocytes compared with high CD11c expression on DC (40, 46, 47). Furthermore, MHC-II expression on Gr-1 high monocytes was greatly increased during infection in an IL-12- and IFN-γ-dependent fashion. In contrast, MHC-II expression on CD11c high DC was very high in both steady-state and infection on CD11c high monocytes is not clear, as both subsets express MHC-II and costimulatory molecules, albeit at much lower levels than DC. Bacterial association bacteria can respond differently than bystander cells exposed only to factors in the environment (47). Bacterial association did not, however, overcome the IFN-γ requirement for recruited monocytes to produce iNOS or up-regulate MHC-II. Thus, the IFN-γ-mediated pathways inducing iNOS and MHC-II up-regulation during infection cannot be overcome by alternate pathways induced in bacteria-containing cells, as can occur for the up-regulation of costimulatory molecules on DC that cannot respond to TNF-α (47).

The Ag presentation capacity of the myeloid lineage cells differed. For example, while recruited (Ly6C high) monocytes induced only marginal proliferation of OT-II CD4 + T cells after stimulation with OVA peptide, Ly6C low monocytes induced robust proliferation. The reason for this differential capacity of Ly6C low and Ly6C high monocytes is not clear, as both subsets express MHC-II and costimulatory molecules, albeit at much lower levels than DC. Effectively inducing T cell proliferation may be a property acquired during monocyte differentiation and is performed only by the mature Ly6C low population. Alternatively, iNOS produced by monocytes could inhibit T cell proliferation (62).

In addition and in contrast to DC, neither Ly6C low nor Ly6C high monocytes caused OT-II proliferation after coincubation with Salmonella expressing OVA. This was somewhat surprising given the capacity of the recruited Ly6C high monocytes to phagocytose Salmonella and up-regulate MHC-II and costimulatory molecules during infection. It could be that the Ag-processing machinery in monocytes is not capable of generating the OVA peptide from internalized Salmonella expressing OVA, at least within the time frame examined here. It has also recently been shown that a subset of DC in PP, those expressing CCR6, are required for CD4 + T cell priming during oral Salmonella infection (28). The activation of OT-II cells by Salmonella-pulsed MLN DC but not monocytes is consistent with the role of CCR6 + DC in priming naïve T cells to iNOS or TNF-α (~30%) make it unlikely that bacterial uptake and destruction (which negates eGFP detection) accounts for the predominance of eGFP + cells making iNOS or TNF-α. The data rather suggest the production of TNF-α and iNOS by bystander cells, an observation consistent with other infection models (5, 12). Cells containing bacteria can respond differently than bystander cells exposed only to factors in the environment (47). Bacterial association did not, however, overcome the IFN-γ requirement for recruited monocytes to produce iNOS or up-regulate MHC-II. Thus, the IFN-γ-mediated pathways inducing iNOS and MHC-II up-regulation during infection cannot be overcome by alternate pathways induced in bacteria-containing cells, as can occur for the up-regulation of costimulatory molecules on DC that cannot respond to TNF-α (47).

The Ag presentation capacity of the myeloid lineage cells differed. For example, while recruited (Ly6C high) monocytes induced only marginal proliferation of OT-II CD4 + T cells after stimulation with OVA peptide, Ly6C low monocytes induced robust proliferation. The reason for this differential capacity of Ly6C low and Ly6C high monocytes is not clear, as both subsets express MHC-II and costimulatory molecules, albeit at much lower levels than DC. Effectively inducing T cell proliferation may be a property acquired during monocyte differentiation and is performed only by the mature Ly6C low population. Alternatively, iNOS produced by monocytes could inhibit T cell proliferation (62).

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We also investigated cell populations in the blood of orally infected mice. Similar to infected tissues, CD68<sup>high</sup>Gr-1<sup>int</sup> monocytes, as well as CD68<sup>high</sup>Gr-1<sup>−hi</sup> neutrophils, were present in the blood of naive mice and increased during infection. The overall phenotype of the blood neutrophils correlated with that in infected PP and MLN except for CD62L, which is down-regulated after infection (64). CD68<sup>high</sup>Gr-1<sup>−lo</sup> monocytes expanded in the PP and MLN except for CD62L, which is down-regulated after infection (64). CD68<sup>high</sup>Gr-1<sup>−lo</sup> monocytes were further activated for, by example, IFN-γ to exhibit these effector functions and also increase CD80 and CD86 expression. The rapid accumulation of CD68<sup>high</sup>Gr-1<sup>−lo</sup> cells in tissues and blood and their capacity to take up bacteria and produce iNOS and TNF-α in tissues, as well as their higher CCR2 and CD62L expression relative to CD68<sup>high</sup>Gr-1<sup>−hi</sup> cells, suggest that CD68<sup>high</sup>Gr-1<sup>−hi</sup> cells are recruited from the blood to PP and MLN and represent the inflammatory monocyte subset (3, 7). There appears to be a developmental relationship between the monocyte subsets, where the Gr-1<sup>−hi</sup> (Ly6<sup>Chigh</sup>) cells are more immature cells recruited to inflamed sites that mature into Gr-1<sup>−lo</sup> (Ly6<sup>C</sup>) monocytes in tissues (8, 25).

MAdCAM-1 is an addressin expressed by high endothelial venules that mediates selective lymphocyte homing into mucosal tissues (45, 56, 57). Blood lymphocytes express the MAdCAM-1 ligands α<sub>β2</sub>, integrin and CD62L, the former mediating homing to PP and the latter to peripheral lymph nodes (65). Because monocytes and neutrophils express CD62L and monocytes also express α<sub>β2</sub> (37, 54, 55), we tested the role of MAdCAM-1 in monocyte and neutrophil recruitment to PP during oral Salmonella infection. However, normal recruitment of these cells was apparent when MAdCAM-1 was blocked. This suggests that pathways other than or in addition to this addressin operate for the entry of monocytes from the blood to PP during oral Salmonella infection. P-selectin can be expressed on high endothelial venules in PP and up-regulated during inflammation (66), and its ligand PSGL-1 is expressed by both monocytes and neutrophils (67). In the absence of MAdCAM-1, P-selectin could thus play a role in the recruitment of monocytes and neutrophils to PP during infection (67, 68).

Consistent with other models (6, 7, 12, 13, 37, 59), our chemo-kine receptor analysis also suggests a critical role of CCR2 in the recruitment of monocytes to Salmonella-infected PP and MLN. Moreover, high CXCR2 expression by the majority of blood neutrophils in infected mice is consistent with its role in recruiting neutrophils to infected lymphoid organs (58). Finally, the presence of CCR6<sup>+</sup> DC in infected PP and MLN is consistent with the role of this DC subset in priming CD4<sup>+</sup> T cells to oral Salmonella infection (28).

Overall, the data presented here provide insight into the relative abundance and function of mononuclear phagocytic populations recruited to infected mucosal lymphoid tissues during oral bacterial infection and in the blood before their arrival in tissues. The data reveal differences in the response and function of phagocytes recruited in the earliest stage of oral Salmonella infection.

**Acknowledgments**

The technical assistance of Kristina Lindgren is acknowledged. We are also grateful to Roy Curtis III (Arizona State University, Phoenix, AZ) for Salmonella χ4550, χ4666, and χ8554, Stephen Schoenberger (La Jolla Institute for Allergy and Immunology, La Jolla, CA) for OT-II mice, Mathias Mack (University of Regensburg, Regensburg, Germany) for anti-CCR2 mAb M21, Arno Hänninen (University of Turku, Turku, Finland) for the MEGA-367 mAb and the supernatant containing the 9B5 mAb, and Mats Bemark for purifying the 9B5 mAb.

**Disclosures**

The authors have no financial conflict of interest.

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