Mycobacterium tuberculosis Recruitment to Lungs Infected with Dendritic Cells Is Crucial for T Cell Dim/Intermediate Macrophages and CD11c Dim/intermediate trafficking of F4/80

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CCR2-Dependent Trafficking of F4/80\textsuperscript{dim} Macrophages and CD11c\textsuperscript{dim/intermediate} Dendritic Cells Is Crucial for T Cell Recruitment to Lungs Infected with \textit{Mycobacterium tuberculosis}\textsuperscript{1}

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We previously reported that CCR2\textsuperscript{−/−} mice are susceptible to \textit{Mycobacterium tuberculosis} infection. Susceptibility was associated with an early and sustained macrophage trafficking defect, followed by delayed recruitment of dendritic cells (DCs) and T cells to the lungs. However, the relative importance of the lack of CCR2 expression by macrophages and DCs vs T cells in susceptibility to infection was unclear. In this study, we used mixed bone marrow transplantation to create mice in which the genotype of the T cells was either CCR2\textsuperscript{+/+} or CCR2\textsuperscript{−/−} while maintaining the genotype of the myeloid cells as CCR2\textsuperscript{+/+}. After infection with \textit{M. tuberculosis}, we found that the genotype of the macrophages and/or DCs, but not that of the T cells, was critical for both T cell and myeloid cell migration to the lungs. Further investigation revealed a critical role for CCR2 in the recruitment of F4/80\textsuperscript{dim} macrophages and CD11c\textsuperscript{dim/intermediate} DCs to the infected lung. \textit{The Journal of Immunology}, 2004, 172: 7647–7653.

E ffective control of \textit{Mycobacterium tuberculosis} infection requires coordination of the innate and adaptive immune responses (see Refs. 1 and 2 for current reviews). Chemokines are secreted proteins that control the trafficking of phagocytic cells and lymphocytes to sites of infection and inflammation as well as through secondary lymphoid tissue (see Refs. 3–5 for current reviews). We recently showed that mice deficient in CCR2 (2), the receptor for monocyte chemoattractant proteins, die soon after infection with \textit{M. tuberculosis}. Infected CCR2\textsuperscript{−/−} mice recruited fewer macrophages, dendritic cells (DCs), and T cells to the lung than wild-type mice, and had delayed production of IFN-γ in the lung and lymph nodes.

Because CCR2 is expressed on immature DCs (6), monocytes/macrophages (7–10), and activated/memory T cells (10, 11), it was not clear whether the susceptibility of the CCR2\textsuperscript{−/−} mice was due to the impaired trafficking of the myeloid cells, T cells, or both. To resolve this issue, we transplanted mixtures of CCR2\textsuperscript{+/+}, CCR2\textsuperscript{−/−}, and TCR-β\textsuperscript{−/−} bone marrow into recombination-activating gene-1 (RAG-1) knockout mice and analyzed immune cell recruitment to the lung after infection with \textit{M. tuberculosis}. In this study, we report that the genotype of the macrophages and/or the DCs, but not of the T cells, is critical for T cell recruitment to the infected lungs.

Materials and Methods

**Mice**

CCR2\textsuperscript{−/−} mice (12) were backcrossed nine times with C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). Littermate CCR2\textsuperscript{−/−} mice were bred to generate the mice used in the experiments. RAG-1\textsuperscript{−/−} and TCR-β\textsuperscript{−/−} mice were from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions and were studied at 6–12 wk of age.

**Generation of bone marrow chimeras**

Mixed bone marrow chimeras were generated with methods adapted from Cyster and Goodnow (13). Four groups of six RAG-1\textsuperscript{−/−} mice were irradiated twice with cesium 137 (500–550 rad each, 4 h apart) from a Mark 1 model 68A irradiator (JL Shepherd and Associates, San Fernando, CA). Various mixtures of bone marrow cells (3 × 10\textsuperscript{6} in 300 μL of PBS) were then injected into the tail vein: 100% C57BL/6 wild-type bone marrow, 100% CCR2\textsuperscript{−/−} bone marrow, 10% wild-type and 90% TCR-β\textsuperscript{−/−} bone marrow, or 10% CCR2\textsuperscript{−/−} and 90% TCR-β\textsuperscript{−/−} bone marrow (Table I). All mice received polymixin B sulfate (85,000 U/L) and neomycin (1100 mg/L) (Sigma-Aldrich, St. Louis, MO) in their drinking water every day for 6 wk while engraftment took place.

**Infection with \textit{M. tuberculosis**}

A virulent strain of \textit{M. tuberculosis} (H37Rv) was grown for stocks after passage through mice. \textit{M. tuberculosis} was titrated using 7H11 agar plates (Fisher Scientific, Springfield, NJ) and stored at −80°C in 1-mL aliquots. Mice were infected via the tail vein with \textit{M. tuberculosis} in 200 μL of PBS and 0.05% Tween 80 (Sigma-Aldrich); bone marrow-transplanted mice were infected with 2 × 10\textsuperscript{6} to 2.5 × 10\textsuperscript{6} CFUs; in experiments comparing CCR2\textsuperscript{−/−} and wild-type mice, the mice were infected with 1 × 10\textsuperscript{6} CFUs; and in experiments using wild-type mice for the CCR2 Ab studies, the mice were infected with 2.3 × 10\textsuperscript{6} CFUs. After each infection, the inoculum was titered (eight, 1/10 dilutions) in PBS and 0.5% Tween 80. The agar plates were incubated at 37°C upside down in ziplock bags, and colonies were counted 3 wk later.

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\textsuperscript{3}Abbreviations used in this paper: DC, dendritic cell; int, intermediate; MCP, monocyte chemoattractant protein; RAG, recombination-activating gene; mid, middle fluorescence.

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Isolation of lung leukocytes

Lungs were excised and placed in 9 ml of digestion buffer (RPMI 1640, 5% FCS, and 1% 10 mM HEPES) (all from Invitrogen, Carlsbad, CA), cut into small pieces (<3 mm³), and digested for 45 min at 37°C in 1 ml of collagenase D and 30 μl of DNase (both 10 mg/ml; Roche, Indianapolis, IN). The tissue suspension was pushed through a 50-μm cell strainer (BD Biosciences, Franklin Lakes, NJ), and the cell suspension was centrifuged for 5 min at 1500 rpm. The collagenase solution was discarded, and the cell pellet was resuspended in fresh digestion buffer. The cells were washed by centrifugation for 5 min at 1500 rpm. The collagenase solution was discarded, and the cell pellet was resuspended in fresh digestion buffer. The cells were washed by centrifugation for 5 min at 1500 rpm, and the red cells were removed by hypotonic lysis for 10 min at room temperature. The cells were washed again, resuspended in 1 ml of staining buffer (PBS, 1% FCS, 0.1% NaN₃, Sigma-Aldrich), and counted with a hemocytometer (14).

Flow cytometry

For bone marrow chimera experiments, 200 μl of lung leukocytes (2.5 × 10⁶ cells/ml) was plated in 96-well V-bottom culture plates and incubated with rat anti-mouse CD16/CD32 (BD Biosciences) (1/500, in staining buffer) for 30 min at 4°C to block Fc receptors. Leukocytes were washed once with staining buffer and then incubated for 30 min at 4°C with fluorescent, cell type-specific Abs: F4/80 PE (1/1000; Caltag Laboratories, Burlingame, CA), CD4 FITC, CD8 FITC, CD69 PE, CD11c PE, and CD19 FITC (1/200; BD Biosciences). Leukocytes were then washed twice with staining buffer and fixed in 4% paraformaldehyde in PBS overnight at 4°C. The next day, the cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data were acquired with CellQuest software (BD Biosciences) and analyzed with FlowJo software (TreeStar, Mountain View, CA). Analysis of double-stained APCs was conducted in two steps to eliminate the interference of the autofluorescent, nonstaining cells. First, the cells were gated in either FL1 or FL2 against an empty fluorescence channel (in this case FL3), and in the second step, the gated populations were analyzed in FL1 vs FL2. This procedure eliminated the autofluorescent, nonstaining cells that would otherwise interfere with the analysis if double-stained APCs were analyzed only in FL1 vs FL2.

For CCR2 staining, wild-type lung leukocytes were plated in 96-well V-bottom plates, as above. Fc receptors were blocked using a mouse anti-mouse CD16 Ab (1/50) (Caltag Laboratories) for 30 min at 4°C. Cells were washed and stained with rat IgG2b anti-mouse CCR2 Ab (1/50) (MC-21) (10) for 30 min at 4°C, and were then washed and incubated with biotinylated mouse anti-rat IgG2b (1/200) (BD Biosciences) for 30 min at 4°C. After washing to ensure removal of excess secondary Ab, the cells were incubated simultaneously with the cell type-specific markers described above, and with streptavidin allophycocyanin (1/1000) (BD Biosciences). Cells were then washed, fixed, and analyzed by flow cytometry, as described above.

Statistical analysis

All statistical analyses were performed using the Mann-Whitney nonparametric test (InStat 3.0; GraphPad, San Diego, CA). Values of less than or equal to 0.05 were considered significant.

Results

T cell recruitment to infected lungs is independent of T cell CCR2 genotype

In earlier studies, we showed that CCR2−/− mice had impaired recruitment of T cells to the lung after infection with M. tuberculosis (14). To determine whether this recruitment defect was dependent upon T cell expression of CCR2, we reconstituted irradiated RAG-1−/− mice with mixtures of bone marrow from either CCR2−/− mice, wild-type mice, or TCR-β6−/− mice. Four groups of mice were generated, as depicted in Table I. Mice in group 1 were reconstituted with 100% wild-type bone marrow, resulting in all of the hemopoietic cells having the CCR2+/− genotype. Mice in group 2 were reconstituted with 100% CCR2−/− bone marrow, resulting in all of the hemopoietic cells having the CCR2−/− genotype. Group 3 mice were reconstituted with 10% wild-type bone marrow and 90% TCR-β6−/− bone marrow. In group 3 mice, all of the T cells were derived from wild-type bone marrow and the macrophages and DCs from both wild-type and the TCR-β6−/− bone marrow; thus, both T cells and myeloid cells were 100% CCR2+/−; these mice served as controls for the group 4 mice, in which the T cell population was reconstituted with bone marrow containing only 10% T cell precursors. Group 4 mice were the experimental group and were reconstituted with 10% CCR2−/− and 90% TCR-β6−/− bone marrow. In this group, the vast majority of the myeloid cells were CCR2+/−, and 100% of the T cells were CCR2−/− because TCR-β6−/− marrow cannot contribute any T cell precursors.

Leukocyte recruitment to the lungs was examined on day 17 after infection (Fig. 1). Mice in groups 1, 3, and 4 recruited CD4 T cells to their lungs equally well, indicating that the CCR2 genotype of the CD4 T cells is not critical in effector cell migration. Similar results were seen for CD8 T cells (Fig. 1). Consistent with our previous results in CCR2−/− mice, we found mice reconstituted with 100% CCR2−/− bone marrow (group 2) had significantly fewer CD4 and CD8 T cells in their lungs at day 17 postinfection, as compared with the mice reconstituted with 100% wild-type bone marrow (group 1) (p = 0.004 and p = 0.01, respectively), as well as significantly fewer macrophages and DCs (p = 0.004; data not shown). Furthermore, there were no significant differences in macrophage and DC recruitment between

![FIGURE 1.](http://www.jimmunol.org/) T cell recruitment to the lungs of bone marrow transplant mice after M. tuberculosis infection. Lung leukocytes were isolated 17 days after infection and stained with Abs to detect CD4 T cells and CD8 T cells by flow cytometry. Group 1 mice were transplanted with 100% wild-type (CCR2+/+) bone marrow. Group 2 mice were transplanted with 100% CCR2−/− bone marrow. Group 3 mice were transplanted with a mixture of bone marrow that was 90% TCR-β6−/− and 10% wild type. Group 4 mice were transplanted with a mixture of bone marrow that was 90% TCR-β6−/− and 10% CCR2−/−. One representative experiment of three similar experiments is shown. **, p < 0.01 (Mann-Whitney nonparametric test; n = 5–6 transplanted mice/group).

### Table I. Protocol for bone marrow transplantation and chimera generation in RAG-1−/− mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor bone marrow</th>
<th>Reconstituted genotype T cells</th>
<th>Myeloid cells</th>
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<tbody>
<tr>
<td>Group 1</td>
<td>100% WT*</td>
<td>100% CCR2+/−</td>
<td>WT</td>
</tr>
<tr>
<td>Group 2</td>
<td>100% CCR2−/−</td>
<td>10% WT</td>
<td>CCR2−/−</td>
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<tr>
<td>Group 3</td>
<td>90% TCR-β6−/−</td>
<td>WT</td>
<td>CCR2−/−</td>
</tr>
<tr>
<td>Group 4</td>
<td>90% TCR-β6−/−</td>
<td>10% CCR2−/−</td>
<td>CCR2+/−</td>
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* WT, wild type.
groups 1 and 4 (data not shown), confirming that the myeloid cells were essentially wild-type, even though 10% of the donor bone marrow was CCR2<sup>−/−</sup>.

**CCR2 is expressed on activated and nonactivated CD4 T cells**

Because CCR2 did not appear to direct T cell migration to the lung, we next asked whether or not CCR2 was preferentially expressed on activated (CD69<sup>+</sup>) T cells. In wild-type mice, both CD4<sup>+</sup>/CD69<sup>+</sup> T cells and CD4<sup>+</sup>/CD69<sup>−</sup> T cells increased in number in the lungs over the course of infection, with a greater increase in the CD69<sup>+</sup> population (Fig. 2A). When we examined these subsets for CCR2 expression, we found that CCR2 was expressed on ~50% of the CD4<sup>+</sup>/CD69<sup>+</sup> activated population between days 9 and 17 after infection (Fig. 3, A, B, and D). The CD4<sup>+</sup>/CD69<sup>−</sup> subset also expressed CCR2, but was slower to up-regulate its expression (Fig. 3, A, C, and D). At each time point after infection, there were significantly more activated (CD4<sup>+</sup>/CD69<sup>+</sup>) T cells expressing CCR2 compared with the nonactivated (CD4<sup>+</sup>/CD69<sup>−</sup>) T cells (p = 0.008, 0.002, and 0.04, respectively) (Fig. 3D). When the CD8<sup>+</sup>/CD69<sup>−</sup> and CD8<sup>+</sup>/CD69<sup>+</sup> T cells were examined, both increased in the lungs over time to similar extents (Fig. 2B). However, in contrast to the CD4 T cells, the CD8 T cell subsets expressed much less CCR2 (10–25%) (Fig. 3E). When CCR2 expression was examined, only at day 13 postinfection there were significantly more CD8<sup>+</sup>/CD69<sup>+</sup> T cells expressing CCR2 compared with the CD8<sup>+</sup>/CD69<sup>−</sup> population (p = 0.002) (Fig. 3E).

**Infected lungs contain two major populations of F4/80-positive cells, one CCR2 positive and one CCR2 negative**

Because CCR2-dependent recruitment of macrophages and/or DCs appeared to control T cell trafficking to the infected lung, we next turned our attention to the myeloid cells. Staining of single-cell suspensions of infected lung tissue revealed two populations of macrophage-like, F4/80-positive cells in wild-type mice: bright and dim (Fig. 4, A, B, and D). Initial experiments compared CCR2 wild-type and CCR2<sup>−/−</sup> mice for recruitment of F4/80<sup>dim</sup> and F4/80<sup>bright</sup> cells (Fig. 4A). Subsequent experiments focused on CCR2 expression of F4/80<sup>dim</sup> and F4/80<sup>bright</sup> cells in wild-type mice (Fig. 4D). When CCR2<sup>−/−</sup> mice were examined, there was a significant reduction in the F4/80<sup>dim</sup> population at each of the time points postinfection compared with wild-type mice (p = 0.03, 0.004, and 0.01) (Fig. 4, A and C). In wild-type mice, F4/80<sup>dim</sup> cells were recruited to the lung in greater numbers than F4/80<sup>bright</sup> cells (Fig. 4, A, B, and D). Up to 60% of the F4/80<sup>dim</sup> cells expressed CCR2, compared with <10% of F4/80<sup>bright</sup> cells (Fig. 4E). Thus, F4/80<sup>dim</sup> cells were recruited to the infected lung and expressed CCR2, whereas the F4/80<sup>bright</sup> cells did not express CCR2 and their population did not significantly increase in size during the course of the infection, suggesting that the F4/80<sup>bright</sup> population is a resident macrophage-like population.

**Infected lungs contain three populations of CD11c-positive cells, each with varying levels of CCR2 expression**

Flow cytometry of cells from the lungs of infected wild-type mice revealed three populations of DC-like, CD11c-positive cells: dim, intermediate (int), and bright (Fig. 5, A, B, and D). In wild-type mice, the number of CD11c<sup>dim</sup> and CD11c<sup>int</sup> cells increased on days 9, 13, and 17, whereas the CD11c<sup>bright</sup> population remained unchanged (Fig. 5, A, B, and D). In CCR2<sup>−/−</sup> mice, the CD11c<sup>dim</sup> population was significantly reduced at day 17 postinfection (p = 0.01) as compared with wild-type mice (Fig. 5, A and C). The CD11c<sup>int</sup> population was also significantly reduced in size on days 13 and 17 postinfection (p = 0.02 and 0.01, respectively) as compared with wild-type mice (Fig. 5, A and C). However, there were no significant differences in the numbers of CD11c<sup>bright</sup> cells in the lungs of the CCR2<sup>−/−</sup> mice and wild-type mice (Fig. 5, A and C). Consistent with the data from the CCR2<sup>−/−</sup> mice, the CD11c<sup>int</sup> subset had the greatest proportion of CCR2-positive cells (Fig.
FIGURE 4. CCR2 expression on F4/80⁺ cells in the lung after M. tuberculosis infection. Lung leukocytes were stained with fluorescent Abs against F4/80 and CCR2. F4/80dim and F4/80bright populations were identified by flow cytometry in wild-type and CCR2⁻/⁻ mice at days 9, 13, and 17 postinfection (A). Representative FACS plots from wild-type (B) and CCR2⁻/⁻ mice (C) at day 17 postinfection. Accumulation of F4/80dim and F4/80bright cells in wild-type lungs before (day 0) and after infection (D). Percentages of these F4/80 subsets expressing CCR2 before and after infection (E). *p ≤ 0.05; **p ≤ 0.01; n = 6 mice per time point; one CCR2⁻/⁻ mouse was used to set CCR2 gates in E.

5E). Although the CD11c dim population was recruited to the greatest extent, only 20–35% of the cells expressed CCR2 (Fig. 5E), although this population was still significantly reduced in size in the CCR2⁻/⁻ mice at day 17 postinfection (Fig. 5, A and C). These data do suggest that chemoattractants other than monocyte chemoattractant proteins may be involved in the recruitment of the CD11c dim population. Like the F4/80bright cell population, CD11c bright cells expressed minimal levels of CCR2 both before and after infection, again suggesting this population is a resident population (Fig. 5E).

Subsets of F4/80 cells are CD11c positive

Because F4/80 and CD11c can sometimes be expressed on the same cell, we performed double-staining experiments to determine whether there was any overlap in the cell populations described above. Because lung cells are highly autofluorescent, we first gated the cells into F4/80dim and F4/80bright populations and then assessed CD11c expression. Only a small proportion of the F4/80dim population (10.2, 21, 37.6, and 23.5%, on days 0, 9, 13, and 17, respectively) expressed CD11c, thus appearing more macrophage like. However, the majority of the F4/80bright cells (75.3, 90.4, 86.6, and 70.9%) were CD11c positive (Fig. 6A). This population of myeloid cells is equally positive for F4/80 and CD11c and appears to be a resident population that does not significantly increase after infection. When the F4/80dim/CD11c⁺ cells were examined for CCR2 expression, we found ~30–40% of them expressed CCR2, while <10% of the F4/80bright/CD11c⁺ cells expressed CCR2 (Fig. 6B). Interestingly, when we examined the F4/80dim/CD11c⁻ cells, ~50–70% of them expressed CCR2, and a similar percentage (30–80%) of F4/80bright/CD11c⁻ cells expressed CCR2 (Fig. 6C). Thus, CCR2 expression appears to correlate more with the F4/80⁺/CD11c⁻ populations.

Two CD11c subsets express F4/80

Next, we assessed CD11c-positive cells for F4/80 expression. First, we gated on the CD11c dim, CD11c int, and CD11c bright cells and asked whether they were F4/80 positive or negative. Again, the high level of autofluorescence prevented us from determining whether they were F4/80 bright or F4/80 dim. Relatively few
CD11c<sup>dim</sup> cells were F4/80 positive (15–30%) (Fig. 7A), making these cells more DC like, whereas a greater number of the CD11c<sup>int</sup> and CD11c<sup>bright</sup> cells were F4/80 positive (40–80%) (Fig. 7A). All of the CD11c<sup>+</sup>/F4/80<sup>+</sup> populations increased in percentage in the lungs up to day 13 postinfection (Fig. 7A). When the CD11c<sup>+</sup>/F4/80<sup>+</sup> subsets were analyzed for their CCR2 expression, we found the CD11c<sup>dim</sup>/F4/80<sup>+</sup> cells expressed the most CCR2 (35–50%), followed by CD11c<sup>int</sup>/F4/80<sup>+</sup> cells (15–30%) (Fig. 7B). Relatively few of the CD11c<sup>bright</sup>/F4/80<sup>+</sup> cells expressed CCR2 (Fig. 7B), a result similar to the F4/80<sup>bright</sup>/CD11c<sup>−</sup> cells. However, more divergence in CCR2 expression was seen when we examined the CD11c<sup>+</sup>/F4/80<sup>+</sup> subsets. In this study, we found that 40–65% of the CD11c<sup>dim</sup>/F4/80<sup>+</sup> population expressed CCR2, while <20% of the CD11c<sup>int</sup>/F4/80<sup>+</sup> and CD11c<sup>bright</sup>/F4/80<sup>+</sup> cells expressed CCR2 (Fig. 7C). Thus, CCR2 expression among the CD11c subsets appears to correlate with the CD11c<sup>int</sup> subset, and of that population the CD11c<sup>dim</sup>/F4/80<sup>+</sup> population has the highest frequency of CCR2.

Discussion

Earlier work from our group and others has demonstrated that chemokine-directed recruitment of lymphocytes and APCs is critical for the host response to infection with <i>M. tuberculosis</i> (14, 15). In the current study, we have used mixed bone marrow transplantation to determine the relative importance of CCR2 expression on T cells vs myeloid cells in this response. There are three major conclusions from this study. First, the CCR2 genotype of the myeloid cells, but not that of the T lymphocytes, is critical for the recruitment of both these cell types to the infected lung. Second, both activated and nonactivated T cells were recruited to the infected lungs, and CD4 T cells expressed higher levels of CCR2 than the CD8 T cells. However, CCR2 expression on T cells was not required for T cell trafficking to the lung, as shown by the bone marrow chimera results. Finally, although several subpopulations of macrophages and DCs were recruited, CCR2 expression was highest on the F4/80<sup>dim</sup> and CD11c<sup>dim</sup> subsets, followed by the CD11c<sup>int</sup> subset.

CCR2<sup>−/−</sup> mice have increased susceptibility to <i>M. tuberculosis</i> and another lung infection, <i>Cryptococcus neoformans</i>, and demonstrate impaired trafficking of leukocytes to the lung and secondary lymphoid tissue (14–16). Because CCR2 is expressed on macrophages, DCs, and T cells, it was unclear whether the increased susceptibility of CCR2<sup>−/−</sup> mice was due to impaired macrophage and DC trafficking and the resultant inability to kill the bacteria and present Ags for T cell activation, or to CCR2-dependent trafficking of the effector T cells to the lung. By varying the ratio of

**FIGURE 7.** CCR2 expression on lung CD11c subsets, positive and negative for F4/80, after <i>M. tuberculosis</i> infection. Lung leukocytes were stained with fluorescent Abs against F4/80, CD11c, and CCR2. Percentages of F4/80<sup>dim</sup>/CD11c<sup>−</sup>, F4/80<sup>int</sup>/CD11c<sup>−</sup>, and F4/80<sup>bright</sup>/CD11c<sup>−</sup> cells before and after infection (A). Percentages of CD11c<sup>dim</sup>/F4/80<sup>−</sup>, CD11c<sup>int</sup>/F4/80<sup>−</sup>, and CD11c<sup>bright</sup>/F4/80<sup>−</sup> cells expressing CCR2 (B). Percentages of CD11c<sup>dim</sup>/F4/80<sup>−</sup>, CD11c<sup>int</sup>/F4/80<sup>−</sup>, and CD11c<sup>bright</sup>/F4/80<sup>−</sup> cells expressing CCR2 (C). n = 6 mice per time point, and one CCR2<sup>−/−</sup> mouse was used to set CCR2 gates.
wild-type, CCR2−/−, and TCR-β8−/− bone marrow donor cells transplanted into irradiated RAG-1−/− mice, we were able to create conditions in which the T cells were either CCR2+/+ or CCR2−/−, while the macrophages and DCs were always CCR2+/+. As shown in Fig. 1, the number of CD4 and CD8 T cells recruited to the infected lung was independent of their CCR2 genotype. In contrast, when both the myeloid cells and T cells were CCR2−/−, far fewer CD4 and CD8 T cells were recruited. These data suggest that CCR2-dependent macrophage and/or DC recruitment to the site of infection and/or to the mediastinal lymph node are critical for recruiting T cells to the lungs. It is possible that APCs secrete chemokines other than the monocyte chemotactic proteins (MCPs) that attract T cells directly to the lungs, or up-regulate chemokine receptors on the T cells while they are in the lymph nodes. Kipnis et al. (17) have recently reported impaired macrophage trafficking to the lung in MCP-1−/− mice, but no decrease in recruitment of activated T cells. Taken together with other data, these results suggest that CCR2 ligands other than MCP-1 may indirectly be critical for T cell recruitment to the lung.

Although CCR2 on T cells does not appear to be critical for T cell recruitment to the lungs, it was expressed on both activated and nonactivated CD4 T cells, and to a much lesser extent on CD8 T cells. The lack of CCR2 requirement for migration, despite T cell expression, has recently been reported in an experimental model of cerebral malaria (18). Similarly, in a model of renal inflammatory disease (19), T cells were found to express CCR2, but also significant levels of CCR5. It thus appears that expression of CCR2 on effector T cells is sometimes redundant and not required for trafficking to inflammatory sites.

Because CCR2-dependent APC trafficking was shown to be critical in both the innate and adaptive immune responses to infection with tuberculosis, we next sought to further define the cell type trafficking to the lung. Two populations of F4/80-positive cells were clearly discernable in the lungs of the infected wild-type mice: F4/80dim and F4/80bright (in our previous paper (14) we measured the lung F4/80dim population only and called it F4/80). After infection with M. tuberculosis, the F4/80dim population increased, in cell number consistent with our previous report (14). In contrast, the F4/80bright population remained unchanged, suggesting that the latter cells were resident interstitial lung or alveolar macrophages. Because CCR2−/− mice failed to recruit F4/80dim cells to the infected lung, we suspected that CCR2 was highly expressed on this population of cells, and this turned out to be correct. On days 0–17 after infection, 50–60% of F4/80dim cells, but only 10% of F4/80bright cells, in the lung were CCR2 positive. These data suggest that F4/80dim, but not F4/80bright, cells are recruited to infected lungs in a CCR2-dependent manner.

Interestingly, the percentages of CCR2+/F4/80− cells were similar before and after infection, suggesting that CCR2 not only recruits specific leukocyte subsets to sites of infection, but is also important in the constitutive trafficking of leukocytes through the lung. This may reflect the fact that pulmonary cells constantly encounter low levels of pathogens or allergens from the environment. It is also interesting that the percentage of CCR2+/F4/80− cells increased only modestly during the infection. CCR2 is rapidly internalized after ligand binding and activation (20), and it is likely that recently recruited cells quickly become CCR2 negative. Therefore, at any point in time, one is looking at the net effect of an increased number of recently recruited CCR2-positive cells (i.e., blood monocytes, which are highly CCR2 positive) and the down-regulation of CCR2 as monocytes differentiate into macrophages. Our data suggest that, in vivo, the rate of CCR2 internalization was approximately equal to the rate of cell recruitment to the infected lung (Figs. 4E; 5E; 6B and C; and 7, B and C).

DC-like cells also trafficked to the lung in response to infection with M. tuberculosis. Three populations of CD11c-positive cells were discernable in wild-type mice: CD11cdim, CD11cint, and CD11cbright (in our previous paper, we grouped the lung CD11cint and CD11cbright population together and called them CD11cbright; however, results reported in this work clearly show three separate CD11c populations). The CD11cdim and CD11cint populations increased dramatically after infection, but the CD11cbright population remained stable, suggesting that these cells are resident pulmonary cells. CCR2 was expressed most intensely on the CD11cint cells, the subset of CD11c-positive cells whose trafficking was most impaired in CCR2−/− mice. Fewer CD11cdim cells expressed CCR2 than the CD11cint population, but still significantly fewer CD11cdim cells were present in the lungs of CCR2−/− mice than wild-type mice at day 17 postinfection. It is possible that CD11cdim cells use multiple chemokine receptors for migration into the lung. The CD11cbright population had the least percentage of CCR2-expressing cells, and consistent with this result there were no significant differences in CD11cbright cells recruited to the CCR2−/− lungs compared with wild-type mice.

Monocytes/macrophages and DCs share a number of properties. Indeed, monocytes are able to differentiate into DCs during the course of Ag presentation and maturation, as discussed by Randolph et al. (21). We therefore asked whether any of the F4/80-positive cells in the lung were also CD11c positive. Most F4/80bright cells were, but the majority of F4/80dim cells were not. Because lung cells are highly autofluorescent, it was difficult to determine whether the F4/80 cells that also stained for CD11c were CD11cdim, CD11cint, or CD11cbright. Interestingly, of the F4/80dim populations, the more macrophage-like F4/80dim/CD11c− cells expressed the most CCR2. Similarly, of the F4/80bright subpopulations, the F4/80bright/CD11c− cells expressed the most CCR2, although there were few of these cells present in the lungs and, thus, they did not contribute to a significant decrease in the F4/80bright population in the CCR2−/− lungs overall. We also performed the converse of this analysis, in which we first grouped the cells as CD11cdim, CD11cint, or CD11cbright and then asked whether they were F4/80 positive or negative. In this case, expression showed considerable overlap between the two markers. CD11cbright cells were almost uniformly F4/80 positive. As discussed above, both CD11cint and F4/80bright cells are probably resident populations, and perhaps the same population. Recently, a study in which APCs released from the lungs of tuberculosis-infected mice were stained for CD11c and CD11b concluded that the CD11cbright/CD11b− and the CD11c−/CD11b+ populations were alveolar macrophages (22). The resident myeloid cell populations we identified appear to be either CD11cbright/F4/80− or F4/80bright/CD11c+. Three-color staining of the lung APCs for CD11c, CD11b, and F4/80 will be required to determine whether these are the same myeloid cell populations described above.

Because these resident myeloid cell populations expressed little or no CCR2, they may use other chemokines and receptors to initially traffic to the lung in a constitutive (noninflammatory) fashion. Recently, Geissmann et al. (23) found two populations of blood monocytes. One is CCR2 positive and CXCR1low and is recruited to inflamed tissues. The other is CCR2 negative and CXCR1bright and is recruited to noninflamed tissues. It will be interesting to determine whether the F4/80bright/CD11c+/CCR2− and the CD11cbright/F4/80+/CCR2− populations of resident cells use CXCR1 to migrate to the lung. CD11cint cells were largely (50–80%) F4/80 positive and may be monocytes/immature DCs. Consistent with this possibility, they expressed the most CCR2 of all the CD11c−/F4/80− populations.
(Fig. 7B) and the most CCR2 of all the CD11c<sup>dim</sup>/F4/80<sup>−</sup> subpopulations (Fig. 7C). It is also possible that these CD11c<sup>dim</sup>/CCR2<sup>−</sup> cells are the CD11c intermediate DCs that produce TNF and inducible NO synthase described by Serbina et al. (24) in the innate immune response to *Listeria monocytogenes*. These DCs were recruited to the livers of infected mice in a CCR2-dependent fashion to kill the bacteria. Further work will be required to ascertain whether this is the same DC population found in lungs infected with *M. tuberculosis*. Gonzalez-Juarrero et al. (22) described a CD11c<sup>dim</sup>/CD11b<sup>−</sup> population as small macrophages and monocytes. Because of the differences in recruitment kinetics and in the expression of F4/80, we believe they are not the same F4/80<sup>dim</sup> cells we identified as macrophages. However, as with the resident myeloid cell populations, three-color staining for CD11c, CD11b, and F4/80 will help to establish the identity of this CD11c<sup>dim</sup> subset.

In conclusion, the recruitment of T cells to the lung after infection with *M. tuberculosis* is not directly mediated by T cell expression of CCR2, but instead requires CCR2-dependent recruitment of macrophages and/or DCs to the lung, and/or to the mediastinal lymph node. Also, of the numerous populations of myeloid cells recruited to the infected lung, CCR2 was highly expressed and required for recruitment by F4/80<sup>dim</sup> macrophage-like cells and CD11c<sup>dim</sup> DC-like cells and to a lesser extent by CD11c<sup>dim</sup> DC-like cells. As potent CCR2 antagonist drugs are introduced into human clinical trials, it will be important to determine whether they increase susceptibility to *M. tuberculosis* or other intracellular pathogens, particularly in immunocompromised patients.

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