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Accumulation of CD11b⁺ Lung Dendritic Cells in Response to Fungal Infection Results from the CCR2-Mediated Recruitment and Differentiation of Ly-6C<sup>high</sup> Monocytes<sup>1,2</sup>

John J. Osterholzer,³,* Gwo-Hsiao Chen,³ Michal A. Olszewski,†# Jeffrey L. Curtis,#,*## Gary B. Huffnagle,‡ and Galen B. Toews*#

Pulmonary clearance of the encapsulated yeast Cryptococcus neoformans is associated with the CCR2-mediated accumulation of lung dendritic cells (DC) and the development of a T1 adaptive immune response. The objective of this study was to identify the circulating DC precursor(s) responsible for this large increase in lung DC numbers. An established murine model was used to evaluate putative DC precursors in the blood, bone marrow, and lungs of CCR2<sup>−/−</sup> mice and CCR2<sup>+/−</sup> mice throughout a time course following infection with C. neoformans. Results demonstrate that numbers of Ly-6C<sup>high</sup> monocytes increased in parallel in the peripheral blood and lungs of CCR2<sup>+/−</sup> mice, whereas CD11c<sup>+</sup> MHC class II<sup>+</sup> pre-DC were 10-fold less prevalent in the peripheral blood and did not differ between the two strains. Accumulation of Ly-6C<sup>high</sup> monocytes correlated with a substantial increase in the numbers of CD11b<sup>+</sup> DC in the lungs of infected CCR2<sup>+/−</sup> mice. Comparative phenotypic analysis of lung cells recovered in vivo suggests that Ly-6C<sup>high</sup> monocytes differentiate into CD11b<sup>+</sup> DC in the lung; differentiation is associated with up-regulation of costimulatory molecules and decreased Ly-6C expression. Furthermore, in vitro experiments confirmed that Ly-6C<sup>high</sup> monocytes differentiate into CD11b<sup>+</sup> DC. Accumulation of Ly-6C<sup>high</sup> monocytes and CD11b<sup>+</sup> DC was not attributable to their proliferation in situ. We conclude that the CCR2-mediated accumulation of CD11b<sup>+</sup> DC in the lungs of Cryptococcus-infected mice is primarily attributable to the continuous recruitment and differentiation of Ly-6C<sup>high</sup> monocytes. The Journal of Immunology, 2009, 183: 8044–8053.

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igration of resident lung dendritic cells (DC) to regional lymph nodes is critical for the initiation of a wide variety of immune responses to microbial pathogens but also to noninfectious inflammatory and allergic stimuli (1–3). In concert with this efflux of DC from the lung, nonresident DC and monocytes accumulate in the lung in response to local microbial or host-derived “danger signals” (3–17). These cell types are critically important for mediating host defense against microbial pathogens yet also contribute to deleterious immune responses as seen in murine models of asthma (18, 19) or the lung injury associated with influenza infection (8, 20). The biological mechanisms responsible for the accumulation of these cells within inflamed lungs are not well understood; knowledge of these mechanisms might result in novel therapies designed to increase (or diminish) lung DC numbers for the treatment of pulmonary disease.

We previously demonstrated that CCR2 mediates the accumulation of large numbers of DC within the lungs of mice infected with Cryptococcus neoformans (7). This encapsulated yeast, acquired by the respiratory tract, causes pneumonia and disseminated infection in immunocompromised patients (21–23) and can also circumvent intact host defenses (24–26). A T1 immune response and classical macrophage activation are essential to clear the organism (27–34). Our results suggest that T1 immune responses are promoted by interactions between nonresident DC and CD4 T cells specifically within newly formed bronchovascular infiltrates. In contrast, mice deficient in CCR2 accumulate few DC within the lung, do not develop T1 immune responses, and fail to clear the organism (7, 35). Thus, this model system is conducive to studies that enhance our understanding of pulmonary adaptive immune responses.

The strong association between the accumulation of lung DC, T1 polarization, and fungal clearance motivated us to further investigate the mechanism(s) responsible for this accumulation of DC in mice infected with C. neoformans. In the prior study, DC accumulation was preceded by an increase in the CCR2 ligands CCL2 (MCP-1) and CCL7 (MCP-3) in the lungs of CCR2<sup>−/−</sup> mice infected with C. neoformans (7). In concert with the profound reduction in lung DC in infected CCR2<sup>−/−</sup> mice, this finding strongly suggests that the CCL2/CCL7/CCR2 axis mediates the recruitment of a circulating DC precursor to the lung.

The circulating precursor that contributes to DC accumulation in response to fungal infection in the lung has not been identified. We
had found that lung DC accumulation in response toinhaled particulate Ag (in mice) is associated with a minor increase in CD11c+/MHC class II+ “pre-DC” in the peripheral blood (13). However, the low percentage of such pre-DC did not readily explain the large increase in the numbers of lung DC. Evidence in other models suggests that DC accumulation in infected peripheral tissues can result from the differentiation of Ly-6Chigh “inflammatory” monocytes (8, 36). In the mouse, these inflammatory monocytes do not express CD11c but strongly express Ly-6C, Gr-1, CD11b, and CCR2. They are distinct (but may be derived from) populations of “resident” or “patrolling” monocytes that express markedly less Ly-6C and CCR2 but strongly express the fractalkine receptor CX3CR1 (37, 38). Thus, Ly-6Chigh inflammatory monocytes could differentiate into lung DC during cryptococcal infection.

The objective of the current study was to identify the circulating precursor of DC recruited to the lungs of mice infected with C. neoformans. Based on our prior results demonstrating that DC accumulation is CCR2 dependent, we predicted that the predominant precursor of lung DC in CCR2+/+ mice would be diminished (or absent) in CCR2−/− mice. We conducted a thorough kinetic analysis identifying DC or their precursors in three tissues compartments to further localize the location of defects leading to impaired lung DC accumulation. We further assessed whether in situ proliferation of DC or their precursors contributed to their overall numbers in the lung.

Materials and Methods

Mice

Specific pathogen-free inbred female BALB/c (designated CCR2+/+) mice purchased from Charles River Laboratories were used, except as specified. CCR2−/− mice (C57BL/6 × J129) backcrossed eight times onto a BALB/c background were provided by W. Kuziel (Molecular Genetics and Microbiology, University of Texas, Austin, TX) (39) and were bred on site. Mice were housed in the animal care facility at the Ann Arbor Veterans Affairs Health System (Ann Arbor, MI) and cared for using a protocol approved by the Veterans Administration’s Institutional Animal Care and Use Committee. Mice were 8–12 wk of age at the time of infection and there were no age-related differences in the responses of these mice to C. neoformans infection.

Cryptococcus neoformans

C. neoformans strain 52D was obtained from the American Type Culture Collection (strain 24067); this strain displayed smooth colony morphology when grown on Sabouraud dextrose agar. For infection, yeast were grown to the stationary phase (48–72 h) at 37°C in Sabouraud dextrose broth (1% 

In vitro culture of Ly-6Chigh monocytes

We performed three types of experiments. First, we compared potential precursors (in the peripheral blood, bone marrow, and lung) of lung DC in CCR2+/+ mice and syngeneic CCR2−/− mice at multiple time points postinfection with C. neoformans. Uninfected CCR2+/+ mice and CCR2−/− mice served as additional controls (designated as day 0). We used four- to six-color flow cytometric analysis on single cell suspensions generated from peripheral blood, bone marrow, or enzymatically digested lung tissue. gating strategies were developed that distinguished subpopulations of potential DC precursors (in peripheral blood and bone marrow) and monocytes and DC (in the lung). Gates were kept consistent in all experiments. This approach permitted the simultaneous quantitative analysis of multiple monocyte cell populations throughout a kinetic time course of C. neoformans infection. Second, we determined the capacity of Ly-6Chigh monocytes to differentiate into DC in vitro. Third, we used a 5-ethyl-2'-deoxouridine (EdU) assay to assess lung and bone marrow tissue for the presence of proliferating DC or monocytes.

Tissue collection

PBMC were isolated from peripheral blood obtained from the retro-orbital vein of deeply anesthetized mice and processed as previously described (13). Lungs were perfused via the right heart using PBS containing 0.5 mM EDTA until pulmonary vessels were grossly clear. Lungs were then excised, minced, and enzymatically digested and a single cell suspension of lung leukocytes was obtained as previously described (7). Bone marrow was harvested by removing the ends from both femurs with a no. 10 blade scalpel (after removal of skin and muscle) and flushing the marrow with 2 ml of PBS (using a 3-cc syringe and a 25.5-gauge needle) into a sterile 70-mm dish. After erythrocyte lysis, cells were washed, filtered over a 70-μm nylon filter, and counted on a hemocytometer, and diluted to 3.3 × 10⁸ CFU/ml in sterile nonpyrogenic saline before intratracheal inoculation. Unencapsulated, heat-killed C. neoformans strain 602 (ATCC strain 62072) was used for the stimulation of in vitro cultures.

Surgical intratracheal inoculation

Mice were anesthetized by i.p. injection of ketamine (100 mg/kg; Fort Dodge Laboratories) and xylazine (6.8 mg/kg; Lloyd Laboratories) and restrained on a small surgical board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A 30-gauge needle was attached to a 1-ml tuberculin syringe filled with diluted C. neoformans culture. The needle was inserted into the trachea, and a 30 μl of inoculum (10⁴ CFU) was dispensed into the lungs. The needle was removed, and the skin was closed with a cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

Monoclonal Abs

The following mAbs purchased from BioLegend were used: N418 (anti-murine CD11c, hamster IgG1); 2G4 (Fc ‘block’); anti-murine CD16/CD32, rat IgG2b); 30-F11 (anti-murine CD45, rat IgG2b); 16-10A1 (anti-murine CD80, hamster IgG2); GL1 (anti-murine CD86, rat IgG2a); MIH5 (anti-murine B7-H1, rat IgG2a); TY25 (anti-murine B7-DC, rat IgG2a); AMS-32.1 (“MHC class II”) (anti-murine I-Ad, mouse IgG2b); 145-2C11 (anti-murine CD3e, hamster IgG1, k); 6D5 (anti-murine CD19, rat IgG2a); and BM8 (anti-murine F4/80, rat IgG2a). The mAbs AL-21 (anti-murine Ly-6C, rat IgM); M1/70 (anti-murine CD11b, rat IgG2b); and 23/3 (anti-murine CD40, rat IgG2a) were purchased from BD Biosciences/BD Pharmingen. The mAbs were primarily conjugated with FITC, PE, PerCP, Cy5.5, allophycocyanin, allophycocyanin-Cy7, or Pacific Blue. Isotype-matched irrelevant control mAbs (BioLegend) were tested simultaneously in all experiments.

Ab staining and flow cytometric analysis

Staining, including blockade of Fc receptors, and analysis by flow cytometry were performed as described previously (40, 41). Data was collected on an LSR II or BD FACS Vantage flow cytometer using FACS Diva software (both from BD Immunocytometry Systems) and analyzed using FlowJo software (Tree Star). A range of cells (10,000–100,000) were analyzed per sample.

Experimental design

To obtain Ly-6Chigh monocytes, we first depleted Ly-6G− cells using a 5-ethyl-2'-deoxouridine (EdU) assay to assess lung and bone marrow tissue for the presence of proliferating DC or monocytes.
an EasySep PE-magnetic selection kit according to the manufacturer’s protocols (STEMCELL Technologies). Ly-6C<sup>high</sup> monocytes were subsequently positively isolated using EasySep FITC anti-Ly-6C-magnetic selection kit. Purified Ly-6C<sup>high</sup> monocytes (~90% purity) were cultured and differentiated at 3 × 10<sup>5</sup> cells/ml in the presence of 20 ng/ml GM-CSF (PeproTech) in complete medium (defined above). The resultant nonadherent cell populations were analyzed after days 3, 5, and 7 in culture (and compared with freshly isolated cells, designated as day 0) by flow cytometry for expression of Ly-6C, CD11c, and CD11b.

In additional experiments, CD11c<sup>/</sup>CD11b<sup>+</sup> cells were assessed for the expression of MHC class II (I-Ab) and costimulatory molecules (CD40, CD80, and CD86). These CD11c<sup>/</sup>CD11b<sup>+</sup> cells resulted from the culture of Ly-6C<sup>high</sup> bone marrow-derived monocytes (in complete medium; defined above) under one of three conditions: 1) GM-CSF alone for 7 days; 2) GM-CSF for 7 days plus 1 μg/ml LPS (Ultra Pure LPS; Escherichia coli O111:B4, catalog no. 421; List Biological Laboratories) on day 6 of culture; or 3) GM-CSF for 7 days plus heat-killed C. neoformans strain 602 (HK-Cneo) at a 1:2 ratio of cells:HK-Cneo on day 6 of culture. Nonadherent cells from all cultures were removed for analysis on day 7.

**Evaluation of monocyte and DC proliferation in vivo**

A Click-iT Edu Pacific blue flow cytometry assay kit (catalog no. A10034) was purchased from Invitrogen for the labeling and detection of DNA synthesis. The labeling strategy used incorporation of the thymidine analog Edu, follow-up by chemical coupling of the analog with an azide-conjugated fluorophore for detection. Mice were injected i.p. either with 100 μg of Edu in PBS or PBS alone (negative control), and after 2 h the mice were sacrificed and bone marrow cells (as positive control) and lung leukocytes were identified by flow cytometric analysis (as described above) for Edu uptake following the manufacturer’s protocols. Gates depicting positive uptake were set based on the staining characteristics of control leukocytes obtained from infected mice not receiving Edu (42, 43).

**Statistical analysis**

All data were expressed as mean ± SEM. Continuous ratio scale data were evaluated by an unpaired Student t test (for comparison between two samples) or by ANOVA (for multiple comparisons) with post hoc analysis by a two-tailed Dunnett test, which compares treatment groups to a specific control group (44). Statistical calculations were performed on a Dell 270 computer using GraphPad Prism version 3.00 for Windows. Statistical difference was accepted at p < 0.05.

**Results**

CCR2 mediates an increase in the frequency of Ly-6C<sup>high</sup> monocytes but not pre-DC within peripheral blood of mice with cryptococcal lung infection

Our previous study demonstrated the accumulation of large numbers of DC in the lungs of CCR2<sup>+/+</sup> mice, but not of CCR2<sup>−/−</sup> mice, infected with C. neoformans (7). The first objective of this study was to look throughout the course of infection for evidence in peripheral blood of two potential DC precursors, pre-DC (defined as CD45<sup>+</sup>CD11c<sup>−/+</sup>-I-Ad<sup>+</sup>) (45–47) and Ly-6C<sup>high</sup> inflammatory monocytes (defined as CD45<sup>+</sup>CD11b<sup>+</sup>Ly-6C<sup>high</sup>), which are known to express CCR2 (37, 38). Our gating strategy involved initial exclusion of debris, RBC, and cell clusters using light scatter, followed by a forward scatter (FSC) vs fluorescence (FL)-4 scatter plot to select for CD45<sup>+</sup> leukocytes (data not shown). To identify pre-DC, we used a FL-8 (CD11c<sup>+</sup>) vs FL-1 (MHC class II) plot (Fig. 1, A and B), whereas analysis of the same samples using a FL-1 (Ly-6C) vs FL-6 (CD11b) plot was used to identify Ly-6C<sup>high</sup>CD11b<sup>+</sup> monocytes (Fig. 1, C and D).

In CCR2<sup>+/+</sup> mice, the pre-DC population increased by only a small amount by day 14 postinfection, relative to uninfected mice (Fig. 1B). In contrast, numbers of Ly-6C<sup>high</sup> monocytes were increased by day 7 postinfection, peaked at day 14, and at all time points were ~10-fold more prevalent than pre-DC (Fig. 1D). In CCR2<sup>−/−</sup> mice, the percentage of pre-DC was low and did not differ significantly from the percentage of pre-DC identified in CCR2<sup>+/+</sup> mice at any time point. The percentage of Ly-6C<sup>high</sup> monocytes in the blood of CCR2<sup>−/−</sup> mice was low in uninfected mice and did not increase following infection (with the exception of a small but statistically significant increase at day 21). Ly-6C<sup>high</sup> monocytes were significantly diminished in CCR2<sup>−/−</sup> mice (relative to CCR2<sup>+/+</sup> mice) at all time points. Collectively, these data show that CCR2 mediates an increase in the frequency of circulating Ly-6C<sup>high</sup> monocytes, but not pre-DC, in mice with cryptococcal lung infection.
therefore evaluated the bone marrow of infected CCR2−/− mice, and it was observed that the decreased frequency of Ly-6C<sup>+</sup> monocytes in the blood of CCR2−/− mice with cryptococcal lung infection appears attributable to impaired egress from the bone marrow and not impaired generation.

**CCR2 mediates the accumulation of large numbers of Ly-6C<sup>+</sup> monocytes in the lungs of mice infected with C. neoformans**

Our next objective was to identify Ly-6C<sup>+</sup> monocytes within the lungs of infected mice. Unless otherwise indicated, all samples were stained with the following Abs: either anti-MHC class II (I-A<sup>+</sup>) (FITC; FL-1) or anti-Ly-6C (FITC; FL-1); anti-F4-80 (PE; FL-2); anti-CD3 plus anti-CD19 (PerCP Cy5.5; FL-3); anti-CD45 (allophycocyanin; FL-4); anti-CD11b (allophycocyanin-Cy7; FL-6); and anti-CD11c (Pacific blue; FL-8). After excluding cell debris, lymphocytes, and eosinophils (based upon their unique FSC<sup>low</sup> vs side scatter (SSC)<sup>high</sup> characteristics) and gating on CD45<sup>+</sup> leukocytes, we focused on the CD11c-negative (CD11c<sup>−</sup>) population (FSC vs FL-8 plot) (Fig. 3A; gate R1). Use of a FL-6 (CD11b) vs FL-1 (Ly-6C) plot allowed us to distinguish CD11c<sup>−</sup> Ly-6C<sup>+</sup>CD11b<sup>+</sup> inflammatory monocytes (gate “Ly-6C<sup>+</sup> mono”, Fig. 3A) (8, 36) from neutrophils, which are CD11b<sup>+</sup>Ly-6C<sup>−</sup>Ly-6C<sup>moderate</sup> cells (Fig. 3A). Ly-6C<sup>+</sup> monocytes coexpressed F4/80 (Fig. 3B) and were medium-sized cells with scant cytoplasm (Fig. 3C).

This gating strategy was then applied to leukocyte populations obtained from the lungs of CCR2<sup>+/+</sup> and CCR2−/− mice at day 0 (uninfected) or days 7, 10, 14, 21, and 28 postinfection with C. neoformans. Consistent with our prior studies (7, 35, 48), C. neoformans infection resulted in massive accumulation of CD45<sup>+</sup> leukocytes in the lungs of CCR2<sup>−/−</sup> mice (peaking at 1.7 ± 0.2 × 10<sup>7</sup> leukocytes per lung at day 14 postinfection) relative to their accumulation in CCR2<sup>+/+</sup> mice (5.9 ± 0.9 × 10<sup>6</sup> leukocytes per lung at day 14 postinfection). Ly-6C<sup>+</sup> monocytes were rare in the lungs of uninfected CCR2<sup>+/+</sup> mice but had significantly increased by day 7 postinfection and peaked at day 14 (6.0 ± 0.1% of total CD45<sup>+</sup> leukocytes; 9.9 ± 0.1 × 10<sup>6</sup> Ly-6C<sup>+</sup> monocytes per lung). This change represents a 54-fold increase relative to uninfected mice (Fig. 3, D and E). In CCR2<sup>−/−</sup> mice, numbers of Ly-6C<sup>+</sup> monocytes were markedly diminished, with the maximal decrease (96%) occurring at day 14 postinfection (Fig. 3D and E).

To verify our findings, we also examined an alternative definition of lung monocytes as CD11c<sup>+</sup> F4/80<sup>+</sup> CD11b<sup>+</sup> cells (F4/80<sup>+</sup> monocytes) (9, 14). Greater than 95% of F4/80<sup>+</sup> lung monocytes in CCR2<sup>+/+</sup> mice expressed high amounts of Ly-6C at days 7, 14, 21, and 28 postinfection (data not shown). F4/80<sup>+</sup> monocyte accumulation was reduced in CCR2<sup>−/−</sup> mice relative to CCR2<sup>+/+</sup> mice at all times postinfection. Overall, the pattern of F4/80<sup>+</sup> monocyte accumulation was very similar to that shown in Fig. 3D for Ly-6C<sup>+</sup> monocytes (data not shown). Collectively, these data indicate that the kinetics of the CCR2-dependent accumulation of Ly-6C<sup>+</sup> monocytes in the lungs during cryptococcal infection closely parallels their increase in peripheral blood.

**CCR2 mediates the accumulation of large numbers of CD11b<sup>+</sup> DC in the lungs of mice infected with C. neoformans**

Our next objective was to identify the predominant DC population in the lungs of CCR2<sup>+/+</sup> mice with cryptococcal infection by using flow cytometric analysis. We used a panel of Abs and gating strategy (described in the preceding section) to first exclude cell debris, lymphocytes, and eosinophils within a population of CD45<sup>+</sup> lung leukocytes (day 14 postinfection). Next, we identified lung macrophages and DC as CD11c-expressing (CD11c<sup>+</sup>) cells

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**FIGURE 2.** Ly-6C<sup>+</sup> monocytes accumulate in the bone marrow of CCR2<sup>−/−</sup> mice with cryptococcal lung infection. Bone marrow cells were obtained from both femurs of CCR2<sup>+/+</sup> and CCR2<sup>−/−</sup> mice 7 days postinfection with C. neoformans. Flow cytometric analysis was used to identify total CD45<sup>+</sup> leukocytes (A) and the frequency (B) and total numbers (C) of pre-DC (CD45<sup>+</sup>CD11c<sup>−</sup>I-A<sup>+</sup>) and Ly-6C<sup>+</sup> monocytes (CD45<sup>+</sup>Ly-6C<sup>+</sup>CD11b<sup>+</sup>). Total numbers of cells were calculated by multiplying the frequency of a cell population by the total number of cells obtained from the bone marrow. Black bars, CCR2<sup>+/+</sup> mice; gray bars, CCR2<sup>−/−</sup> mice. Data represent mean ± SEM of 3–4 mice per strain assayed individually per time point; †, p < 0.05 by unpaired Student t test. These time courses were independently repeated once in their entirety (n = 3 mice per time point) with similar results.
FIGURE 3. CCR2 mediates the accumulation of Ly-6Chigh monocytes in the lungs of mice with cryptococcal infection. A, Gating strategy to identify Ly-6Chigh monocytes in the lung using flow cytometric analysis (representative plots of CCR2+/+ mice on day 7 postinfection): After exclusion of cell debris, lymphocytes, and eosinophils, a plot of CD11c vs FSC identifies CD11c-negative cells (left dot plot; gate R1). Within this population, Ly-6Chigh monocytes are identified as Ly-6ChighCD11b+ cells (right dot plot; gate Ly-6Chigh mono). B, Ly-6Chigh monocytes express F4/80 (shaded histogram, isotype control; open histogram, specific Ab staining). C, Morphology of Ly-6Chigh monocytes (sorted using the above gating strategy; H&E staining and ×1,000 original magnification). D and E, Flow cytometric analysis of lung leukocytes from CCR2+/+ and CCR2−/− mice at day 0 (uninfected) or days 7, 10, 14, 21, and 28 days after intratracheal (IT) infection with C. neoformans (Data Days Post Cneo IT). D, Representative dot plots (day 7) identifying Ly-6Chigh monocytes within lung leukocytes obtained from CCR2+/+ mice (left dot plot) and CCR2−/− mice (right dot plot). E, Total numbers of Ly-6Chigh monocytes (calculated by multiplying the frequency of Ly-6Chigh monocytes by the total number of CD45+ lung leukocytes at each time point. Data in E represent mean ± SEM of 3–6 mice (black solid lines, CCR2+/+ mice; gray dashed line, CCR2−/− mice) assayed individually per time point; *p < 0.05 by ANOVA with Dunnet’s post hoc analysis vs day 0 (uninfected) of mice of the same CCR2 expression profile; ‡, p < 0.05 by unpaired Student t test between CCR2+/+ vs CCR2−/− mice at the same time point. These time courses were independently repeated once in their entirety (n = 3 mice per time point) with similar results.

(FSC vs FL-8 plot) (Fig. 4A, left dot plot, gate R2). We then used autofluorescence (FSC vs FL-3 plot) (Fig. 4A, middle dot plot) to distinguish autofluorescent (AF)-positive (AF+) macrophages from nonautofluorescent (AF−) DC (7, 49, 50). Within the nonautofluorescent gate (Fig. 4A, middle dot plot; gate R2a), a FL-6 (CD11b) vs FL-8 (CD11c) plot was used to identify a dense population of CD11c+ AF+ CD11b+ DC (Fig. 4A, right dot plot, gate “DC”). CD11b+ DC expressed MHC class II and F4/80 (Fig. 4, B and C) and CD11b+ DC sorted from lung leukocytes using this gating strategy displayed characteristic cytoplasmic extensions and ruffled cell membranes (Fig. 4D). They did not express CD103 (data not shown), distinguishing them from the small population of AF− CD11b+ CD103− DC described in the airways of uninfected
mice (51). CD11b+ DC also did not express B220, distinguishing them from plasmacytoid DC (data not shown).

We applied this gating strategy to assess the frequency and total number of CD11b+ DC within the lungs of individual CCR2+/+ mice or CCR2−/− mice at baseline and at multiple time points following infection with C. neoformans (Fig. 4E). The percentages and total numbers of lung CD11b+ DC increased significantly in the lungs of infected CCR2+/+ mice, peaking at day 14 postinfection (10.7 ± 0.1% of total CD45+ leukocytes, 17.7 ± 0.1 × 106 CD11b+ DC per lung; Fig. 4E). In CCR2−/− mice, total numbers of lung CD11b+ DC were diminished (96%), most notably at day 14 postinfection (Fig. 4E). These data demonstrate the CCR2 dependence of CD11b+ DC accumulation during cryptococcal lung infection.

**Comparative phenotypic analysis of Ly-6C^high monocytes and CD11b+ lung DC in vivo**

The strong temporal correlation between the accumulation of Ly-6C^high monocytes and CD11b+ DC in CCR2+/+ mice suggested that Ly-6C^high monocytes might differentiate into CD11b+ DC within the lungs. To assess this hypothesis, we compared the two cell types for their scatter properties and expression of MHC class II and costimulatory molecules. Ly-6C^high monocytes were smaller (FSClow) than CD11b+ DC and had less granular cytoplasm (SSClow) (Fig. 5A). Ly-6C^high monocytes expressed moderate amounts of MHC class II and minimal amounts of CD40, CD80, CD86, and B7-H1. They did not express B7-DC (Fig. 5B, top histograms). In contrast, CD11b+ DC strongly expressed MHC class II and a modest amount of the costimulatory molecules CD40, CD80, and CD86 (Fig. 5B, bottom histograms). Furthermore, CD11b+ DC express moderate to high amounts of B7-H1 and B7-DC.

Reports suggest that differentiation of Ly-6C^high monocytes results in down-regulation of Ly-6C (52, 53). Therefore, we assessed CD11b+ lung DC for their expression of Ly-6C on days 7, 14, and 21 postinfection (in CCR2+/+ mice) (Fig. 5C). CD11b+ lung DC expressed high amounts of Ly-6C at day 7 postinfection, whereas expression was lower on CD11b+ DC in the lung at days 14 and 21. Collectively, our data suggested that Ly-6C^high monocytes differentiate into CD11b+ DC within the lung; their differentiation is associated with the following phenotypic changes: 1) up-regulation of CD11c; 2) an increase in size and in the cytoplasm:nuclear ratio; 3) enhanced expression of MHC class II and costimulatory molecules; and 4) down-regulation of Ly-6C.

**Ly-6C^high monocytes differentiate into CD11b+ DC in culture**

To verify the capacity of Ly-6C^high monocytes to differentiate into CD11b+ DC, we purified Ly-6C^high monocytes from the bone marrow of uninfected CCR2+/+ mice and examined the nonadherent population at various times during in vitro culture for up to 7 days in GM-CSF (20 ng/ml) (Fig. 6). Freshly isolated Ly-6C^high monocytes were small cells with little cytoplasm. Following 7 days in culture the cells were larger, contained more cytoplasm, and displayed cytoplasmic extensions and ruffled cell membranes consistent with DC morphology. Flow cytometric analysis confirmed that cell size (FSC) and granularity (SSC) increased over time (data not shown). Ly-6C expression decreased over time, whereas by day 7 the majority of cells coexpressed CD11c and CD11b (Fig. 6A). These CD11c+CD11b+ cells expressed MHC class II (I-A^d) and costimulatory molecules (CD40, CD80, and CD86; Fig. 6B, top histograms). Expression of these molecules increased further in response to stimulation with heat-killed C. neoformans (Fig. 6B, middle histograms) or LPS (Fig. 6B, bottom histograms). These results demonstrate that Ly-6C^high monocytes can differentiate in vitro into DC displaying a phenotype similar to that identified in vivo for CD11b+ DC in the lungs of mice with cryptococcal lung infection.
CD11b+ DC and Ly-6C<sup>high</sup> monocytes are not proliferating in the lungs of mice infected with C. neoformans

To test the possibility that accumulation of CD11b+ DC could also result in part from the proliferation of DC (or their precursors) within the lung, we treated CCR2<sup>+/+</sup> mice with the thymidine analog EdU to identify proliferating cells by flow cytometric analysis (42, 43, 54). We evaluated leukocytes obtained from the bone marrow and lungs 10 days postinfection (Fig. 7). This time point was chosen because our kinetic analysis revealed that CD11b+ DC (or their precursors) within the lung, we treated CCR2<sup>+/+</sup> mice with the thymidine analog EdU. Two hours later, bone marrow and lung leukocytes were removed and specific cell populations were assayed (by flow cytometric analysis, see Materials and Methods and gating strategies described in Figs. 3 and 4) for evidence of EdU uptake (as an indicator of cell proliferation). Representative histograms of bone marrow (A) and lung (B) leukocyte populations. Shaded histograms, EdU staining performed on leukocytes from infected control mice not receiving EdU administration; open histograms, EdU staining performed on leukocytes from infected mice receiving EdU administration. Gates indicate positive EdU staining. The experiment was performed three times with similar results. Ly6C<sup>mod</sup>, Ly6C moderate.

**Table I. Proliferation of bone marrow and lung leukocytes in CCR2<sup>+/+</sup> mice**

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Positive EdU (±SEM)</th>
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<td>Bone marrow Total cells</td>
<td>16.6 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt; monocytes</td>
<td>19.9 ± 1.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ly-6C&lt;sup&gt;mod&lt;/sup&gt; PMN</td>
<td>11.0 ± 1.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung CD11b+ DC</td>
<td>−0.1 ± 0.3</td>
</tr>
<tr>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt; monocytes</td>
<td>−0.6 ± 2.0</td>
</tr>
<tr>
<td>Ly-6C&lt;sup&gt;mod&lt;/sup&gt; PMN</td>
<td>−0.1 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells identified by flow cytometric analysis as per Materials and Methods. Data represent percentage of cells staining for EdU (mean ± SEM of three mice assayed individually per time point).

<sup>b</sup> p < 0.05 by an unpaired Student t test comparing EdU expression from EdU-treated mice vs untreated (but infected) controls; the experiment was repeated with similar results.

**Discussion**

This study is the first to define the mechanisms responsible for the sizeable accumulation of CD11b+ DC in the lungs of mice following pulmonary infection with a fungal pathogen. Collectively, our data imply that the accumulation of CD11b+ lung DC in mice with cryptococcal infection results from the continuous CCR2-mediated recruitment and differentiation of Ly-6C<sup>high</sup> monocytes. The following important observations support this conclusion: 1) CCR2 expression was strongly associated with an increase in the
percentage of Ly-6C<sup>high</sup> monocytes, but not pre-DC, in the peripheral blood of infected mice; 2) this observed increase in Ly-6C<sup>high</sup> monocytes within peripheral blood was paralleled by their substantial accumulation in the lungs of CCR2<sup>+/+</sup> mice (but not CCR2<sup>−/−</sup> mice); 3) CD11b<sup>+</sup> DC were also strikingly more abundant in the lungs of infected CCR2<sup>+/+</sup> mice than in those of CCR2<sup>−/−</sup> mice, and a comparative phenotypic analysis suggests that Ly-6C<sup>high</sup> monocytes differentiate into CD11b<sup>+</sup> DC in vivo; 4) Ly-6C<sup>high</sup> monocytes differentiate into CD11b<sup>+</sup> DC in vitro; and 5) neither CD11b<sup>+</sup> DC nor Ly-6C<sup>high</sup> monocytes proliferated within the lung.

The clear demonstration that Ly-6C<sup>high</sup> monocytes increase in the blood of CCR2<sup>+/+</sup> mice during cryptococcal lung infection implies that the lung communicates with the bone marrow, likely via a systemic signal. The onset of accumulation of Ly-6C<sup>high</sup> monocytes in both blood and lungs on day 7 in the current study coincides with the timing we previously found for pulmonary expression of two CCR2 ligands, CCL2 (MCP-1) and CCL7 (MCP-3) (7). The marked sequestration of Ly-6C<sup>high</sup> monocytes in the bone marrow of infected CCR2<sup>−/−</sup> mice suggests that CCR2 facilitates monocyte egress from the bone marrow, as proposed by other investigators (36, 56, 57). Local production of CCR2 ligands within the bone marrow could contribute to this process (58). This collective evidence indicates that the peripheral pulmonary microenvironment facilitates the release of additional monocytes from the bone marrow and that the CCL2/CCL7/CCR2 axis is a critical mediator of this important process.

In contrast to our data for Ly-6C<sup>high</sup> monocytes, the percentage of pre-DC (MHC class II<sup>−</sup>/CD11c<sup>−</sup>) in the peripheral blood did not differ between CCR2<sup>+/+</sup> and CCR2<sup>−/−</sup> mice infected with C. neoformans. This is the first study to demonstrate that their presence in the peripheral blood (in the resting state or in response to infection) is CCR2 independent. The frequency of pre-DC in PBMC did increase, albeit slightly, in both strains at day 14 postinfection. This finding was comparable to the small increase in circulating pre-DC we reported in wild-type C57BL/6 mice challenged with inhaled particulate Ag (13). The contribution of these cells to total DC accumulation in the lungs in either the former or current study is unclear. Importantly, however, they were 10-fold less prevalent than Ly-6C<sup>high</sup> monocytes in both the blood and bone marrow of CCR2<sup>−/−</sup> mice (in this study). These findings lead us to conclude that pre-DC are not major contributors to the total population of CD11b<sup>+</sup> lung DC following C. neoformans infection.

Results of our study show that lung infection with a fungal pathogen is a potent stimulus for the differentiation of Ly-6C<sup>high</sup> monocytes into CD11b<sup>+</sup> DC. Data from several of our experiments support this conclusion. First, our kinetic analysis in CCR2<sup>+/+</sup> mice reveals a strong association between the recruitment of Ly-6C<sup>high</sup> monocytes and the accumulation of lung CD11b<sup>+</sup> DC. Second, our comparative phenotypic analysis of these cells recovered from the lung suggests that Ly-6C<sup>high</sup> monocytes do the following: 1) enlarge; 2) up-regulate the expression of CD11c, MHC class II, and costimulatory molecules; and 3) decrease the expression of Ly-6C as they differentiate into CD11b<sup>+</sup> DC. These findings are in agreement with others documenting the loss of Ly-6C expression over time and as monocytes differentiate (8, 52, 53). Third, results from our culture of Ly-6C<sup>high</sup> monocytes verify that these cells differentiate into CD11c<sup>+</sup>CD11b<sup>+</sup> cells in vitro. Their resultant expression of costimulatory molecules was similar to that observed for CD11b<sup>+</sup> lung DC; expression of these molecules was further enhanced by exposure to Cryptococcus.

Our conclusion that most lung CD11b<sup>+</sup> DC in murine cryptococcal lung infection differentiated from Ly-6C<sup>high</sup> monocytes differs from reports suggesting that CD11b<sup>+</sup> lung DC are derived from Ly-6C<sup>low</sup> monocytes (59, 60). Disparity in experimental approaches may explain this difference and provide additional insight into factors that influence monocyte differentiation. The studies concluding that CD11b<sup>+</sup> DC result from differentiated Ly-6C<sup>low</sup> monocytes evaluated monocytes differentiation either in the resting state (59), following the ablation of resident cells (60), or in response to a single challenge with LPS (59, 60). Results of those studies indicate that the replacement of resident DC or the accumulation of DC in response to brief inflammatory stimuli can be achieved via the differentiation of Ly-6C<sup>low</sup> monocytes. In contrast, we evaluated the response to pulmonary challenge with a live fungal pathogen. The resultant immune response is massive, with upward of 150 million leukocytes recruited to the lung. Defense against the organism occurs over weeks and may be influenced by multiple pathogen-associated molecular patterns and virulence factors (4, 61–64). We cannot exclude the possibility that some CD11b<sup>+</sup> lung DC in our study may have differentiated from Ly-6C<sup>low</sup> monocytes. Yet, our observation that >95% of F4/80<sup>+</sup> monocytes identified in the lungs of Cryptococcus-infected (CCR2<sup>+/+</sup>) mice expressed high amounts of Ly-6C suggests that few Ly-6C<sup>−/low</sup> monocytes were present (relative to the large number of Ly-6C<sup>high</sup> monocytes). Collectively, these results imply that Ly-6C<sup>high</sup> monocytes are the dominant monocyte subset contributing to lung CD11b<sup>+</sup> DC in response to the intense and prolonged inflammation induced by a replicating pathogen.

The findings that neither CD11b<sup>+</sup> DC nor Ly-6C<sup>high</sup> monocytes were proliferating in the lungs but that Ly-6C<sup>high</sup> monocytes clearly did proliferate in the bone marrow is evidence that proliferation and differentiation of Ly-6C<sup>high</sup> monocytes is compartmentalized. Collectively, our data emphasize the important relationship that develops between the lung and bone marrow in response to pulmonary infection. The CCL2/CCL7/CCR2 axis is actively involved in the early phases of the response and mediates the egress of additional Ly-6C<sup>high</sup> monocytes into the circulation. The role of CCR2 or other chemokine receptors in facilitating monocyte recruitment directly into the lung continues to be investigated (10, 65, 66). Mechanisms involved in resolution of pulmonary inflammation remain poorly understood; however, our observation that CD11b<sup>+</sup> lung DC decrease after day 14 postinfection coincides with a decrease in Ly-6C<sup>high</sup> monocytes in the peripheral blood. This finding suggests that resolution may be closely linked to the signals that mediate the release of Ly-6C<sup>high</sup> monocytes.

Comparison of our findings with those obtained using other model systems demonstrates that the infecting microbe influences the magnitude, cellular phenotype, and microanatomic composition of the CCR2-mediated immune response in the lung. First, our data show that upward of 10 million monocytes and 15 million DC accumulate in the lungs of CCR2-expressing mice infected with C. neoformans. This robust accumulation of these cells is 5- to 100-fold greater than that in studies defining a role for CCR2 in mediating monocyte or DC recruitment to the lung in response to bacterial (6, 9, 67), viral (8, 20, 68), or other fungal (69) pathogens. Second, DC in the lungs of Cryptococcus-infected (CCR2-expressing) mice expressed B7-H1 and B7-DC. Expression of these molecules has not been previously reported on DC recovered from mice infected with a fungal pathogen. These molecules may have immunomodulatory properties and it will be important to determine whether they prevent immune-mediated lung injury, as can occur in cryptococcal and influenza infection (8, 20). Third, use of our model reveals an important link between CCR2-mediated pulmonary DC accumulation and the formation of neolymphoid structures in the lungs. We previously reported that recruited DC colocalize with CD4<sup>+</sup> T cells specifically within neolymphoid...
aggregates (which we termed “bronchovascular infiltrates”) in the lungs of mice infected with *C. neoformans* (7). These structures were diminished in infected CCR2-deficient mice. Similar structures were recently described (as “tertiary lymphoid organs”) in the lungs of mice infected with the influenza virus (70). DC were critical for the formation of these structures and essential for the development of B cell-mediated immune responses against influenza. Thus, this murine model of cryptococcal lung infection highlights both important differences and similarities in the CCR2-mediated development of host defense against a broad array of microbial infections in the lung.

In summary, these data reveal an important role for recruited Ly-6C<sup>high</sup> monocytes in the accumulation of CD11b<sup>high</sup> lungs of mice infected with the influenza virus (70). DC were diminished in infected CCR2-deficient mice. Similar structural mechanisms (including the CCL2/CCL7/CCR2 axis) in mediating their egress from bone marrow, recruitment into peripheral tissue, and differentiation thereafter might have important implications for novel therapeutics designed to enhance or diminish the number of DC in a variety of pulmonary diseases.

**Disclosures**

The authors have no financial conflict of interest.

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