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Tuberculosis Triggers a Tissue-Dependent Program of Differentiation and Acquisition of Effector Functions by Circulating Monocytes

Markus Sköld and Samuel M. Behar

The origin and function of the different myeloid cell subsets that appear in the lung during pulmonary tuberculosis are unknown. Herein we show that adoptively transferred monocytes give rise to many of the macrophage and dendritic cell (DC) subsets that appear following aerosol infection with virulent Mycobacterium tuberculosis. Monocyte differentiation in infected peripheral tissue is surprisingly heterogeneous and results in the formation of five distinct myeloid subsets, including both classically activated macrophages, that produce inducible NO synthase via an IFN-γ-dependent mechanism, and DC. In contrast, monocytes recruited to draining pulmonary lymph nodes are functionally different and acquire a mature DC phenotype. Thus, while monocytes are recruited to the lungs of uninfected mice, their differentiation and acquisition of myeloid effector functions are dramatically altered in the presence of inflammation and bacteria and are dependent on tissue localization. Therefore, our results support a model in which recruited monocytes are well poised to influence multiple aspects of host immunity to infections in the lungs. This report provides the first direct evidence for monocyte differentiation into both the macrophage and DC lineages in vivo following infection with a live human pathogen. The Journal of Immunology, 2008, 181: 6349–6360.
intact hosts (15–18). Surprisingly, concurrent differentiation of monocytes into Mφ has not been observed unless other myeloid cells are selectively depleted, suggesting that this process is regulated (15). Models of sterile inflammation, such as instillation of LPS or sterile thioglycolate (TG) medium, cannot mimic the myriad of host–microbe interactions that eventually shape the host’s immune response during live infection. While few studies have directly monitored monocyte recruitment and differentiation in vivo during infection, Leon et al. identified several monocyte-derived DC subsets in vivo after Leishmania major infection (19). These data challenge the dogma that monocytes recruited to sites of infection differentiate into Mφ.

In this report we provide direct evidence that circulating monocytes are the direct precursors of Mφ and DC found in infected lung and draining pulmonary lymph node (PLN) tissue during pulmonary tuberculosis. We find that monocyte differentiation in lung tissue is dramatically affected by Mtb infection, which causes recruited inflammatory monocytes to differentiate into at least five phenotypically distinct myeloid Mφ or DC subsets. One population of monocyte-derived Mφ has an unusual phenotype characterized by the expression of markers associated with DC. This cell population is the major producer of iNOS in vivo after Mtb infection. Additionally, we find that inflammatory monocytes recruited to the PLN differentiate into cells with a mature DC phenotype, rather than differentiating into Mφ. These findings reveal that monocyte differentiation into the Mφ or DC lineages is dependent on tissue localization. Our results support a model in which the plasticity of monocyte differentiation allows recruited monocytes to be a major source of the multiple myeloid effector cells that are present in the lungs and lung-associated lymphoid tissues during infectious or inflammatory conditions.

Materials and Methods

**Mice and Mtb aerosol infection**

C57BL/6J (B6), B6.SJL-Ptprc<sup>−/−</sup>Pepck<sup>−/−</sup>BoyJ (B6.CD45.1), B6.129S1-C<sup>−</sup>C<sup>−</sup>O<sup>−/−</sup>Ly6a<sup>−/−</sup>Ly6b<sup>−/−</sup>Il2rgtm1Lm1J (CCR2<sup>−/−</sup>), B6.129S1-C<sup>−</sup>C<sup>−</sup>O<sup>−/−</sup>Ly6a<sup>−/−</sup>Ly6b<sup>−/−</sup>Il2rgtm1Lm1J (IL-3R<sup>−/−</sup>/β2<sup>−/−</sup>), B6.129S7-flk10<sup>tm1Ajt</sup> (IFN-γ<sup>−/−</sup>), and C3HeB/FeJ mice were obtained from The Jackson Laboratory. CCR2-deficient (CCR2<sup>−/−</sup>) mice on the B6 genetic background were a kind gift from Dr. Glenn Dranoff (Dana-Farber Cancer Institute, Boston, MA) (22). Monocytes were cultured for 3 days at 37°C, 5% CO₂ to differentiate Mφ and DC. Adherent (detached using 0.05% EDTA) and nonadherent cells were harvested and reseeded into 96-well or 24-well plates, or analyzed using flow cytometry as described below.

**Mixed leukaemia reaction**

Monocyte-derived B6 Mφ and DC were harvested, irradiated, and used as APC in a MLR to measure DC function. Mφ and DC were seeded in complete culture medium in flat-bottom 96-well plates at the indicated cell concentrations (six replicas per condition). Splenic T cells from naive uninfected C3HeB/FeJ mice were enriched using CD90 microbeads (Miltenyi Biotech) and used as effector cells (3 × 10<sup>5</sup> T cells/well). The cocultures were incubated at 37°C, 5% CO₂, and pulsed with [3H]thymidine for 6 h on day 5 to measure T cell proliferation.

**Analysis of iNOS expression and NO production in vitro**

iNOS and NO production was examined as a measurement of Mφ function. Monocyte-derived Mφ and DC were harvested and seeded in flat-bottom 96-well plates (5 × 10<sup>4</sup> cells/well for the NO assay), or in 24-well plates (5 × 10<sup>5</sup> cells/well for iNOS expression) in complete culture medium with 10% L929 supernatant (100 μg/ml), or GM-CSF (12 ng/ml) and recombinant IL-4 (10 ng/ml) (DC). Additionally, the cells were cultured with recombinant IFN-γ (10 U/ml, United States Biological) and the indicated concentrations of ultrapure TLR4 ligand Escherichia coli LPS (0111:B4 strain) from InvivoGen. Adherent and nonadherent cells were harvested after 24 h and analyzed for iNOS expression using flow cytometry as described below. NO<sub>2</sub> in the culture supernatants after 48 h was measured using Griess reagent.

**Monocyte recruitment and differentiation using a model of sterile inflammation**

Monocyte recruitment and differentiation within inflamed tissue were examined using a protocol adapted from Geissmann et al. (16). Local inflammation was induced in either B6.CD45.1 or B6 mice by i.p. injection of 1 ml sterile 3% TG medium (Remel). Untreated primary B6 monocytes (0.5–1 × 10<sup>6</sup>), or CFSE-labeled (Molecular Probes) B6 monocytes (0.5–1 × 10<sup>6</sup>) were adoptively transferred i.v. via the tail vein 6 h later. Monocytes adoptively transferred into naive B6.CD45.1 or B6 mice were used as controls. Three recipient mice were used per group in three separate experiments. Peritoneal exudate cells (PEC) were harvested by lavage and single-cell suspensions were prepared from lung tissue after 18 or 96 h. Mononuclear cells were prepared from lung tissue as described (23). Recruitment and differentiation of CD45.2<sup>+</sup> or CFSE-labeled transferred cells in lung tissue and total PEC were analyzed using flow cytometry as described below. All recipient mice were analyzed individually.

**Monocyte adoptive transfer into Mtb-infected recipient mice**

An adoptive transfer system was used to examine monocyte recruitment, differentiation, and function during pulmonary tuberculosis. B6 monocytes were obtained from naive B6, β<sup>−/−</sup>R<sup>−/−</sup>, IL-3R<sup>−/−</sup>/β2<sup>−/−</sup>, IFN-γ<sup>−/−</sup>, or CCR2<sup>−/−</sup> mice were used as a source of primary monocytes in the monocyte adoptive transfer experiments described below. Uninfected or Mtb-infected B6.CD45.1 mice were housed in a biosafety level 3 facility at the Animal Biohazard Containment Suite (Dana-Farber Cancer Institute, Boston, MA) were used as recipient mice in the monocyte adoptive transfer experiments, and were used in an approved protocol. Mice were infected with virulent Mtb (Erdman strain) via the aerosol route as described previously (21).

**Monocytes and in vitro cultures**

Primary monocytes were enriched from BM of naive donor mice. Donor mice were euthanized using CO₂ and total BM was collected from femurs and tibiae. Monocytes were first enriched by density centrifugation using the Lymphocyte-Mammal reagent (Cedarlane Laboratories). The remaining cells were labeled with unconjugated primary rat IgG Mab (10 μg/ml) to mouse CD31 (MEC 13.3), B220 (RA3-6B2), Ly6G (1A8), and TER-119 (TER-119) (all from BD Pharmingen) and CD90.2 (AT15.E) and I-A<sup>ε</sup>/I-E<sup>ε</sup> (M5/114.15.2) that were purified according to standard procedures. The cells were then incubated with goat anti-rat IgG microbeads (Miltenyi Biotech) to allow for negative selection of monocytes using magnetic cells sorting with LD separation columns (Miltenyi Biotec). Negatively selected cells were 92.6 ± 2.7% (n = 13) monocytes (Ly6C<sup>−</sup> CD11b<sup>−</sup> CD11c<sup>−</sup>) as determined by flow cytometry.

Peripheral blood was obtained by cardiac puncture, and monocytes were enriched by treating the blood with the Lymphocyte-Mammal reagent.

Enriched BM monocytes (5 × 10<sup>5</sup>) were seeded into 24-well plates in complete culture medium (RPMI 1640 (Invitrogen) supplemented with 10% FCS (HyClone), penicillin/streptomycin, t-glutamine, sodium-pyruvate, 2-ME, nonessential amino acids, essential amino acids, and HEPES buffer (all from Invitrogen)). The media were supplemented with 40% L929 supernatant as a source of M-CSF to differentiate Mφ, or with GM-CSF (12 ng/ml) and recombinant IL-4 (10 ng/ml, R&D Systems) to generate DC. GM-CSF producing B16 cells were used as a source of GM-CSF and was a kind gift from Dr. Glenn Dranoff (Dana-Farber Cancer Institute, Boston, MA) (22). Monocytes were cultured for 3 days at 37°C, 5% CO₂ to differentiate Mφ and DC. Adherent (detached using 0.05% EDTA) and nonadherent cells were harvested and reseeded into 96-well or 24-well plates, or analyzed using flow cytometry as described below.

**Flow cytometry**

PBS with 1% (w/v) BSA and 2 mM Na<sub>2</sub>CO<sub>3</sub> was used to wash the single-cell suspensions and to dilute Abs and second step reagents. Cells were incubated with purified anti-CD16/CD32 (2.4G2, BD Pharmingen) at 25 μg/ml to block nonspecific staining. The following FITC-, PE-, Pe-CPE-, PE-Cy<sub>7</sub>-, allophycocyanin-, or allophycocyanin-Cy<sub>7</sub>-conjugated or biotinylated mAbs and second step reagents were obtained from BD Pharmingen: anti-CD1d (1B1), anti-CD3e (145.2C11), anti-CD11b (M1/70), anti-CD11c
Activated CD11b⁺CD11c⁺ Mφ are the main cellular source of iNOS in the lung following Mtb infection

Induction of iNOS expression and NO production is required for control of bacterial growth following infection with aerosolized Mtb. Immunohistochemistry has demonstrated the close proximity of iNOS producing cells to Mtb containing lesions in the lung tissue (24). Despite the importance of NO in protection against Mtb, the origin of these iNOS⁺ cells has remained obscure. Cell surface expression of CD11b and CD11c has been used as a first step to profile the heterogeneous myeloid cell subsets found in lung tissue following pulmonary Mtb infection (Fig. 1A). These studies were complemented by a thorough analysis of various Mφ, DC, and granulocyte markers to characterize the different myeloid cell subsets (Fig. 1B).

Cell surface expression of CD11b and CD11c identified multiple myeloid cell subsets in lung tissue from uninfected mice, none of which contained significant numbers of iNOS⁺ cells (Fig. 1A).
We identified two CD11b<sup>+</sup>CD11c<sup>-</sup> subsets in uninfected lungs: one expressing intermediate CD11b levels, and the other high CD11b levels. These populations consisted of small cells (based on low forward light scatter (FSC) profile) and included both F4/80<sup>-</sup> (low cell surface expression levels, data not shown) and Ly6G<sup>+</sup> cells (Fig. 1), suggesting a mixture of monocytes/M<sub>Φ</sub> and granulocytes. In contrast, the CD11b<sup>int</sup>CD11c<sup>-</sup> cells were predominantly F4/80<sup>-</sup> and Ly6G<sup>+</sup>, and were previously identified as monocytes and small M<sub>Φ</sub> (Fig. 1) (3). The CD11b<sup>-</sup>CD11c<sup>-</sup> cells did not express F4/80 or Ly6G, but were uniformly MHC class II<sup>+</sup> and are resident DC. Finally, the CD11b<sup>hi</sup>CD11c<sup>-</sup> cells are mostly large (FSC<sub>high</sub>), and although many of the cells were positive for F4/80, MAC-3, and MHC class II, the cell surface levels were low (Fig. 1B and data not shown). Most of these cells have the same phenotype as alveolar M<sub>Φ</sub> obtained by bronchoalveolar lavage, but also contain CD11b<sup>low</sup>CD11c<sup>-</sup> DC (3, 25–27).

Within 3 wk of infection with aerosolized M<sub>b</sub>, the absolute number of cells obtained from the lung increased more than 5-fold, due in large part to the development of inflammatory infiltrates consisting predominantly of myeloid and lymphoid cells (Fig. 1B and data not shown) (2, 3). This is accompanied by a change in the CD11b/CD11c profile of the myeloid cells, which may represent differentiation of resident tissue M<sub>Φ</sub> and DC and/or recruitment and differentiation of progenitor cells, including peripheral blood monocytes. In infected lung tissue, two CD11b<sup>+</sup>CD11c<sup>-</sup> subsets were identified. The CD11b<sup>hi</sup>CD11c<sup>-</sup> cells were nearly all Ly6G<sup>-</sup>F4/80<sup>-</sup> granulocytes, while the CD11b<sup>int</sup>CD11c<sup>-</sup> cells contained small (FSC<sub>low</sub>) Ly6G<sup>+</sup>F4/80<sup>-</sup> cells that were monocytes and M<sub>Φ</sub>. In contrast to the uninfected state, most of the CD11b<sup>hi</sup>CD11c<sup>-</sup> cells obtained from infected lung tissue expressed MHC class II. While CD11b<sup>int</sup>CD11c<sup>-</sup> cells from uninfected lung tissue were mostly alveolar M<sub>Φ</sub>, this subset was not readily apparent in M<sub>b</sub>-infected lung tissue. Instead, the CD11b<sup>-</sup>CD11c<sup>-</sup> cells found in infected lung tissue were MHC class II<sup>+</sup> and a significant proportion expressed the DC marker CD83. We did not detect high CD115 (M-CSF receptor) or CD8α expression by any myeloid subset in naive or infected lung tissue (data not shown).

Two discrete populations were identified in the lungs of infected mice that were absent or not abundant in uninfected mice. They were both large (FSC<sub>high</sub>) CD11b<sup>+</sup>CD11c<sup>-</sup> cells that expressed F4/80, MAC-3, and MHC class II (Fig. 1B). The dominant CD11b<sup>+</sup>CD11c<sup>-</sup> population expressed slightly higher CD11b levels, and intracellular iNOS was detected within 44% of these cells, making this subset the principal producers of iNOS in infected lung tissue (Fig. 1A). The other CD11b<sup>-</sup>CD11c<sup>-</sup> population expressed slightly higher CD11c levels but did not produce iNOS. This other population of cells is likely to be alveolar M<sub>Φ</sub> that have up-regulated CD11b in response to inflammation, as described following LPS instillation or Streptococcus pneumoniae infection (15, 28). In conclusion, the iNOS-producing cells found in the lungs of M<sub>b</sub>-infected mice were large CD11b<sup>-</sup>CD11c<sup>-</sup> cells expressing F4/80, MAC-3, and MHC class II, a phenotype consistent with activated M<sub>Φ</sub>, and we hypothesize that these cells are recruited to the lung during M<sub>b</sub> infection. We sought to develop an adoptive transfer model that could be used to study the differentiation and function of these cells in M<sub>b</sub>-infected mice.

**Distinct programs of monocyte differentiation are triggered by cell recruitment to the normal lung vs the inflamed peritoneum.**

We developed an adoptive transfer model using untreated primary BM monocytes to determine which of the myeloid cell subsets identified in the lungs of M<sub>b</sub>-infected mice are derived from monocytes. BM monocytes were chosen because sufficient numbers could be obtained for experimental use. The cell surface phenotype of BM monocytes is similar to peripheral blood monocytes and they have the potential to differentiate into functional M<sub>Φ</sub> or DC as determined by NO production upon activation and the ability to prime naive T cells in an MLR assay, respectively (data not shown). These features of BM monocytes provided a rationale for their use in our adoptive transfer experiments.

Untreated B6 (e.g., CD45.2<sup>+</sup>) BM monocytes were adoptively transferred i.v. into B6.CD45.1<sup>+</sup> recipient (“untreated”) mice. Some mice were injected i.p. with sterile TG medium 6 h before cell transfer (“TG treated”). Monocyte recruitment and differentiation in the peritoneal cavity and in recipient lung tissue were analyzed 96 h after monocyte adoptive transfer. A. Transferred cells were identified in PEC and in lung tissue based on CD45.2 expression compared with an isotype control mAb (data not shown). The number indicates the percentage of gated CD45.2<sup>+</sup> events. B. Transferred cells in elicited PEC and lung tissue were analyzed for expression of the indicated cell surface markers and FSC-side scatter profile. The contour plots show one representative experiment out of three. Numbers indicate percentage of cells in each gate or quadrant.

**FIGURE 2.** Monocyte homing and differentiation are altered by tissue inflammation. B6.CD45.2<sup>+</sup> monocytes were adoptively transferred i.v. into B6.CD45.1<sup>+</sup> recipient (“untreated”) mice. Some mice were injected i.p. with sterile TG media 6 h before cell transfer (“TG treated”). Monocyte recruitment and differentiation in the peritoneal cavity and in recipient lung tissue were analyzed 96 h after monocyte adoptive transfer. A. Transferred cells were identified in PEC and in lung tissue based on CD45.2 expression compared with an isotype control mAb (data not shown). The number indicates the percentage of gated CD45.2<sup>+</sup> events. B. Transferred cells in elicited PEC and lung tissue were analyzed for expression of the indicated cell surface markers and FSC-side scatter profile. The contour plots show one representative experiment out of three. Numbers indicate percentage of cells in each gate or quadrant.
These lung-homing monocytes acquire a strikingly different phenotype from typical inflammatory M\(^+\)H9278. These results show monocytes are recruited to both normal and inflamed tissues, and depending on the local tissue environment, undergo a different program of cell differentiation. We next addressed how pulmonary inflammation associated with tuberculosis alters this program of differentiation.

**FIGURE 3.** Recruited monocytes differentiate within the lung of Mtb-infected mice. Monocytes from B6, CCR2\(^{-/-}\), β2R\(^{-/-}\), IL-3R\(^{αγ}/βcR\(^{-/-}\), or IFN-γR\(^{−/−}\) donor mice were adoptively transferred into Mtb-infected CD45.1\(^+\) congenic recipient mice (3 wk postinfection). Transferred cells in lung tissue were analyzed 4 days later. A. Transferred cells were identified based on CD45.2 expression (upper left panel) compared with an isotype control mAb (lower left panel). Gated CD45.2\(^+\) cells were then analyzed for CD11b and CD11c expression profile (upper right panel) compared with isotype control stainings (lower right panel). Transferred B6 cells are shown in A. B. CD11b and CD11c expression displayed by transferred B6 and knockout monocytes in infected lungs. The bars show means ± SD (n = 3–12). C. Transferred cells recruited to infected lung tissue that were CD11b\(^+\)CD11c\(^−\) (left panel) or CD11b\(^+\)CD11c\(^+\) (right panels) were analyzed for MHC class II and MAC-3 expression. The bars show means ± SD (n = 3–12); **, p < 0.001 by one-way ANOVA with Bonferroni posttest. MAC-3 expression (thick lines) by CD11b\(^+\)CD11c\(^+\) WT and IFN-γR\(^{−/−}\) transferred cells is shown in the histograms (right panels, thin lines indicate isotype control). D. Displayed are MFI of MHC class II (the mAb M5/114.15.2 was either Alexa Fluor 647- or allophycocyanin-conjugated) and MAC-3 (PE-conjugated) expression on gated CD11b\(^+\)CD11c\(^−\) (left panels) and CD11b\(^+\)CD11c\(^+\) (right panels) monocyte-derived cells. The bars show mean ± SD (n = 3–10).

in mice injected i.p. with TG. These lung-homing monocytes acquire a strikingly different phenotype from typical inflammatory Mφ. These results show monocytes are recruited to both normal and inflamed tissues, and depending on the local tissue environment, undergo a different program of cell differentiation. We next addressed how pulmonary inflammation associated with tuberculosis alters this program of differentiation.

**Transferred monocytes are recruited and differentiate in the lungs of mice with established Mtb infection**

We used our monocyte adoptive transfer model to determine whether monocytes are the precursors of Mφ or DC subsets found in the lung during pulmonary tuberculosis. B6 CD45.2\(^+\) monocytes were adoptively transferred into intact B6,CD45.1 congenic recipient mice with established pulmonary tuberculosis (3 wk after aerosol infection). Lung tissue was analyzed 4 days after monocyte adoptive transfer. Transferred cells were identified based on CD45.2 expression (Fig. 3A). To determine the differentiation program of donor monocytes trafficking to infected lung tissue, we analyzed their CD11b and CD11c expression. Two major populations were identified: a population of CD11b\(^+\)CD11c\(^−\) subset consisting of monocytes and small Mφ, and a new population notable for the expression of high CD11c levels, which accounted for most donor cells in the lung (Fig. 3A).
We previously showed that adoptively transferred Mφ traffic to the lungs of Mtb-infected mice where they up-regulate the Ag-presenting molecules CD1d and class II MHC in an IFN-γ-dependent manner (23). To determine whether monocyte differentiation into CD11b⁺CD11c⁺ cells was IFN-γ-dependent, we transferred monocytes obtained from IFN-γ receptor knockout (IFN-γR⁻/⁻) mice into intact recipient mice. Both wild-type (WT) and IFN-γR⁻/⁻ monocytes gave rise to CD11b⁺CD11c⁺ cells when transferred into Mtb-infected mice (Fig. 3B). The donor-derived CD11b⁺CD11c⁻ cells express low levels of MHC class II and MAC-3, and no statistically significant differences were detected between WT and IFN-γR⁻/⁻ monocytes (Fig. 3, C and D). In contrast, the CD11b⁺CD11c⁺ cells significantly up-regulated MHC class II and MAC-3, which is expressed on both Mφ and DC (Fig. 3, C and D). While CD11b⁺CD11c⁺ cells derived from both WT and IFN-γR⁻/⁻ monocytes up-regulated MHC class II, the cells derived from IFN-γR⁻/⁻ monocytes expressed significantly less MAC-3 (Fig. 3C). In particular, development of MAC-3⁺⁺⁺ cells was dependent on IFN-γR (Fig. 3C). However, the mean fluorescence intensity (MFI) of the MAC-3 staining on total CD11b⁺CD11c⁺ cells derived from IFN-γR⁻/⁻ monocytes was not statistically different compared with WT cells (Fig. 3D).

The role of other cytokines and chemokines in monocyte recruitment to the lung and their differentiation in inflamed tissue were investigated using monocytes obtained from cytokine and chemokine receptor knockout mice (Fig. 3B–D). The CCR2 chemokine receptor is expressed by myeloid cells and binds to the family of monocyte chemoattractant protein (MCP) chemokines, of which MCP-1 is the main ligand (29). The greater susceptibility of CCR2 knockout mice to Mtb infection correlates with reduced numbers of Mφ and DC in infected lung tissue (2). Following adoptive transfer of WT or CCR2 knockout monocytes, similar numbers of transferred cells were detected in the lungs of infected mice (data not shown).

Similarly, monocytes were obtained from donor mice lacking the common β-chain of the IL-3, IL-5, and GM-CSF receptor (βR⁻/⁻) that are unresponsive to the cytokines IL-5 and GM-CSF, or IL-3Rβ⁻/⁻/βR⁻/⁻ double-knockout monocytes that fail to respond to IL-3, IL-5, or GM-CSF (Fig. 3B–D) (30, 31). While IL-3, IL-5, and GM-CSF are all important for myeloid cell growth and differentiation, our results show that they are not required for monocyte recruitment and differentiation in the lungs of Mtb-infected recipient mice (Fig. 3B–D). Furthermore, neither CCR2, βR, nor IL-3Rβ/βR appeared to be required for MHC class II and MAC-3 expression (Fig. 3, C and D).

These results demonstrate that when monocytes in circulation home to Mtb-infected lung tissue, they differentiate into cells that express high surface levels of CD11c, MHC class II, and MAC-3, and these events are largely independent of CCR2, IL-3, IL-5, and GM-CSF. While IFN-γ was not required for CD11c and MHC class expression, this proinflammatory cytokine was needed to induce high cell surface expression of MAC-3 on CD11b⁺CD11c⁻ cells.

Monocytes recruited to the lungs and PLN of Mtb-infected mice persist long-term and acquire a stable phenotype

To determine the durability of monocyte-derived cells in the lungs and PLN of uninfected and infected mice, and to assess whether any of these cells undergo terminal differentiation in vivo, we measured changes in the donor cell population over time (Fig. 4). CD11c up-regulation was detected on monocytes homing to the lung within 2 days of their transfer into uninfected recipient mice.
By day 4, two monocyte-derived populations were present: CD11b⁺ CD11c⁻ cells and CD11b⁺CD11c⁺ cells. The frequency of cells in each subset remained relatively constant from day 4 and through day 14 after cell transfer (Fig. 4, A and B). Both subsets up-regulated MHC class II, which also remained relatively constant over the day 4–14 time interval. CD11b⁺CD11c⁺ cells expressed higher cell surface levels of MHC class II compared with CD11b⁺CD11c⁻ cells as determined by MFI of the MHC class II staining. By day 10, CD11b⁺CD11c⁻ cells began to appear in the uninfected lung tissue. However, because these cells were rare, they were not analyzed further (Fig. 4A).

Monocytes transferred into Mtb-infected mice behaved differently. CD11b⁺ monocytes trafficking to infected lung tissue more rapidly up-regulated CD11c, which was expressed at levels that were nearly 10-fold higher compared with uninfected lungs (Fig. 4A). In addition to CD11b⁺CD11c⁺ and CD11b⁺CD11c⁻ cells, a third monocyte-derived subset, which expressed CD11c but not CD11b, began to appear by day 6 after transfer (Fig. 4, A and C). All three monocyte-derived subsets up-regulated MHC class II in infected lung tissue, and the expression levels remained constant between day 4 and day 14 (Fig. 4C). In particular, the CD11b⁺CD11c⁺ cells and the CD11b⁺low CD11c⁻ cells were MHC class IIhigh compared with CD11b⁺ CD11c⁻ cells in infected lungs (Fig. 4C) and monocyte-derived cells in uninfected lung tissue (Fig. 4B). Similar events were detected in the PLN of infected mice (Fig. 4A). Not only did high CD11c levels rapidly appear on the transferred cells in the PLN, but also the monocyte-derived CD11b⁺lowCD11c⁺ cells were even more prominent (Fig. 4, A and D). Particularly at the late time points, the PLN were relatively enriched for monocyte-derived CD11b⁺lowCD11c⁺ cells (Fig. 4, A and D).

In summary, transferred monocytes give rise to three distinct myeloid cell subsets defined by their CD11b and CD11c expression, all of which persist long-term in Mtb-infected lung and PLN tissue. The kinetic analysis presented herein supports a linear differentiation model in which CD11b⁺CD11c⁺ cells generate CD11b⁺lowCD11c⁺ cells, which eventually give rise to CD11b⁻lowCD11c⁺ cells. Although it is not certain that the cells detected in the PLN trafficked first through the lung, the PLN contains the highest proportion of CD11b⁺low CD11c⁺ cells, which resemble DC phenotypically (and see below). Furthermore, these cells are unlike the monocyte-derived peritoneal Mϕ that are formed in response to TG treatment (Fig. 2 and data not shown). Thus, the type of inflammation and the tissue localization modulate the differentiation program of monocytes.

**Adoptively transferred monocytes differentiate into myeloid cell populations identified in the lungs of Mtb-infected mice**

The dramatic increase in myeloid cells found in the lungs following Mtb infection and the appearance of functionally different Mϕ
and DC subsets could be explained by cell recruitment or activation and differentiation of resident tissue Mφ and DC (Fig. 1). Based on CD11b and CD11c profiling, transferred monocytes recruited to the lung differentiate into cells that resemble the population of myeloid cells found in the lungs of Mtb-infected mice (compare Figs. 1 and 4). These results prompted us to analyze in greater detail the monocyte-derived populations identified by CD11b and CD11c profiling in uninfected lung (subsets A and B) and in Mtb-infected lung (subsets C, D, and E) (Fig. 5).

Ten days after monocyte transfer into uninfected mice, the CD11b^hi CD11c^int cells (subset A) lacked expression of MAC-3, CD8α, CD86, and CD83 (Fig. 5A). These cells expressed low levels of MHC class II and F4/80 and represent undifferentiated monocytes or Mφ. Similarly, the CD11b^int CD11c^int monocyte-derived cells (subset B) were MAC-3^−, CD8α^−, CD86^−, and CD83^−, but expressed higher cell surface levels of F4/80 and MHC class II.

Monocyte transfer into Mtb-infected recipients gave rise to three distinct monocyte-derived subsets (Fig. 5B). Like CD11b^hi CD11c^int cells in uninfected lung tissue, CD11b^hi CD11c^− cells in infected lungs (subset C) did not express MAC-3, CD8α, CD86, or CD83. While F4/80 expression was low, MHC class II expression was significantly higher than subset A in uninfected recipients. Finally, CD11b^int CD11c^− cells expressed heterogeneous cell surface levels of Gr-1, suggesting that this subset contains undifferentiated inflammatory monocytes. The monocyte-derived cells that up-regulated CD11c (subset D) expressed MHC class II, MAC-3, F4/80, and CD86, but remained CD8α^− and CD83^− and down-regulated Gr-1. These cells were reminiscent of activated donor Mφ. The last monocyte-derived subset that appeared after cell transfer into infected recipients was CD11b^int CD11c^− (subset E) (Figs. 4 and 5B). These cells expressed less MAC-3 and F4/80 than the CD11b^hi CD11c^− cells, and they remained negative for CD8α and Gr-1. In addition to expressing high levels of MHC class II and CD86, a subset of the CD11b^hi CD11c^− cells were CD86^hi CD83^−, suggesting that these cells were DC (Fig. 5B).

These results confirm that monocytes recruited to the lungs of Mtb-infected mice undergo a complex program of differentiation that generates many of the different types of myeloid cells that are identified in the lungs of infected mice (compare with Fig. 1). The differences we observe in uninfected recipients compared with Mtb-infected mice show that monocyte differentiation is dramatically altered during inflammatory or infectious conditions.

Monocyte-derived cells with a DC phenotype are selectively enriched in the PLN draining infected lung tissue

The distal alveoli are the initial site of infection following aerosolization of Mtb. The bacteria appear first in the PLN before disseminating systemically, and DC play an important role in transporting live bacteria from lung tissue to the draining PLN (8, 21). The PLN is also where the Mtb-specific adaptive immune response is first detected and DC are required for initiating adaptive immunity (8, 21, 32). Based on our finding that cells monocyte-derived CD11b^hi CD11c^− cells become a prominent population in the PLN, we wanted to determine whether these cells resemble DC.

Using CD11b and CD11c profiling, we identified four main populations of resident (CD45.1^+) myeloid cell subsets in the PLN of Mtb-infected mice, which we compared with CD45.2^+ monocyte-derived cells (subsets 1–4, Fig. 6A). CD11b^hi CD11c^− cells (subset 1) expressed intermediate levels of CD86 and low levels of CD83, MAC-3, and MHC class II and likely represent recruited monocytes. CD11b^hi CD11c^− cells (subset 2) were mostly neutrophils (83.6 ± 6.5% Ly6G^+, data not shown). The CD11b^hi CD11c^+ cells (subset 3) expressed higher levels of CD86, MAC-3, and MHC class II than did CD11b^hi CD11c^− cells. Importantly, this subset included nearly all of iNOS-producing cells in the PLN.
Finally, CD11b<sup>int</sup>CD11c<sup>- </sup> cells (subset 4) did not express iNOS or M<sup>AC-3</sup>, but expressed the highest levels of CD86, CD83, and MHC class II, a phenotype consistent with mature DC.

We then characterized the cells derived from adoptively transferred monocytes 10 days after transfer into Mtb-infected mice. Nearly all of the monocyte-derived cells that were found in the PLN had up-regulated CD11c, MHC class II, and M<sup>AC-3</sup> (Figs. 4 and 6 and data not shown). Additionally, these cells expressed heterogeneous CD11b levels, were small (FSC<sup>low</sup>), and did not produce iNOS (Figs. 4 and 6, A and B). Instead, a third of these cells had a distinct CD86<sup>high</sup>CD83<sup>-</sup> phenotype, similar to the resident cells belonging to subset 4, suggesting that these cells are mature DC (Fig. 6, B and C).

These results show that monocytes can serve as progenitor cells to the myeloid cell compartment in PLN draining the lungs during pulmonary tuberculosis. While monocytes do not differentiate into iNOS<sup>+</sup> cells in the PLN, the monocyte-derived cells that are recruited to the PLN acquire a phenotype typical of mature DC.

Monocytes traffic to the lungs of Mtb-infected mice and differentiate into CD205<sup>+</sup> M<sup>φ</sup> that produce iNOS via an IFN-γ-dependent mechanism

Pathogens trigger the production of iNOS by activated M<sup>φ</sup>. Following Mtb infection, the main iNOS-producing cell in the lung is a large CD11b<sup>+</sup>CD11c<sup>+</sup> MHC class II<sup>+</sup> M<sup>φ</sup> (see Fig. 1). We tested the hypothesis that these iNOS<sup>+</sup> M<sup>φ</sup> are derived from monocytes recruited to the lung using our monocyte adoptive transfer model. Monocytes from uninfected B6 CD45.2<sup>+</sup> donor mice were adoptively transferred into uninfected or Mtb-infected (week 3 postinfection) CD45.1<sup>+</sup> congenic recipients. Similarly, monocytes from IFN-γR<sup>-/-</sup> donors (CD45.2<sup>+</sup>) were transferred to Mtb-infected CD45.1<sup>+</sup> recipient mice to determine the role of IFN-γ in iNOS production. Ten days after monocyte adoptive transfer, CD45.2<sup>+</sup> cells detected in uninfected recipient mice could be divided into CD11b<sup>+</sup>CD11c<sup>+</sup> cells (subset A) and CD11b<sup>+</sup>CD11c<sup>-</sup> cells (subset B) (Fig. 7A). As previously demonstrated, three monocyte-derived subsets were identified in the lungs of Mtb-infected recipient mice: CD11b<sup>+</sup>CD11c<sup>-</sup> cells (subset C), CD11b<sup>-</sup>CD11c<sup>-</sup> cells (subset D), and CD11b<sup>+</sup>CD11c<sup>-</sup> cells (subset E) (Fig. 7A). In agreement with our earlier results, monocyte differentiation into these three subsets in Mtb-infected lung tissue was independent of IFN-γ (Figs. 3 and 7A).

These different monocyte-derived subsets were further analyzed for their ability to produce iNOS (Fig. 7, B and C). Neither subset of monocyte-derived cells found in the lungs of uninfected mice made intracellular iNOS, which is consistent with our analysis of the endogenous cell populations (Fig. 1A). In contrast, when transferred into infected mice, WT monocytes trafficked to the lung and differentiated into iNOS-producing cells. While some iNOS was expressed in small CD11b<sup>+</sup>CD11c<sup>-</sup> cells, the chief producers of iNOS were the large CD11b<sup>+</sup>CD11c<sup>-</sup> cells. The monocyte-derived CD11b<sup>-</sup>CD11c<sup>-</sup> cells are small cells that do not produce iNOS, demonstrating that these cells are functionally different as well as phenotypically distinct (Figs. 4 and 5). In contrast to WT monocytes, none of the cell subsets derived from IFN-γR<sup>-/-</sup> monocytes produced iNOS (Fig. 7, B and C). Thus, while IFN-γ is not required for many aspects of monocyte differentiation, including CD11b, CD11c, and MHC class II regulation, these results confirm a critical role for IFN-γ in iNOS production by monocyte-derived cells.

To characterize the iNOS-producing cells further, we measured their CD205 expression. CD205 is a multilectin receptor that is expressed by foamy M<sup>φ</sup> in lung Mtb granulomas (9). As expected, the CD11b<sup>+</sup>CD11c<sup>-</sup> cells lacked CD205 expression, while 65% of monocyte-derived DC (subset E; CD11b<sup>-</sup>CD11c<sup>-</sup>) expressed CD205. Interestingly, among the monocyte-derived CD11b<sup>-</sup>CD11c<sup>-</sup> cells, CD205 expression correlated with the ability of the cells to produce iNOS (Fig. 7D). Only 20% of the iNOS<sup>-</sup> expressed CD205, whereas 81% of the iNOS-producing cells were CD205<sup>+</sup>, which was similar to endogenous iNOS<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> M<sup>φ</sup> (data not shown).

Discussion

The inflammatory response that follows Mtb infection involves a dramatic increase in the total myeloid cell number as well as an increase in the diversity of myeloid lineage cells in the lung and in PLN draining the lung tissue. Activated M<sup>φ</sup> are among the myeloid cells that localize to infected lung tissue, often in close proximity to the Mtb lesions. iNOS expression and NO production by these effector cells are required for control of bacterial growth and to ameliorate disease. To directly address the potential role of monocytes as precursors for activated M<sup>φ</sup> and DC subsets in infected and inflamed tissues, we developed a monocyte adoptive...
transfer model. This model allowed us to track the fate of monocytes at the single cell level in uninfected mice and in mice with pulmonary tuberculosis. Our results reveal that tissue localization, and infection and inflammation, leads to remarkable differences in monocyte differentiation and function. Other progenitor cells exist that could potentially serve the same or similar function as we have described for BM monocytes. Fogg et al. recently identified a CX₃CR1⁺ CD117⁺ CD11b⁺ progenitor of peripheral tissue Mφ and DC designated as Mϕ and DC progenitor (33). These progenitor cells have similar differentiation potential as monocytes, but they may have a different pattern of recruitment to various tissues than we described for BM monocytes (17). Additionally, because it is not known if resident tissue Mϕ and DC are able to divide or further differentiate, we do not know the relative contribution that recruited monocytes make to the myeloid cell compartment in native or infected peripheral tissues and lymphoid organs. However, it is clear from the data presented herein that they have the potential to make a significant contribution to this process and give rise to all of the main myeloid subsets found in lung and PLN tissue during tuberculosis, and provide direct evidence that monocytes are progenitor cells for multiple and functionally distinct Mϕ and DC subsets that are recruited to the lung after infection.

A significant proportion of recruited monocytes in both uninfected and infected lung tissue remained CD11c⁻ and expressed low levels of F4/80, MAC-3, MHC class II, CD83, and CD86. The heterogeneous Gr-1 expression profile by these cells in infected lungs suggests that this subset is comprised of a mixture of Gr-1⁺ inflammatory monocytes and differentiating monocytes, similar to the resident Gr-1⁺ monocyte subset present in peripheral blood. Alternatively, the CD11b⁺ CD11c⁻ cells may be differentiated cells expressing cell surface markers that were not tested in the present study (e.g., PDCA-1, a plasmacytoid DC marker). How is this cell population maintained over time? A recent study by Varol et al. showed that BM monocytes adoptively transferred i.v. are able to migrate into the BM and later reenter the circulation (17). This property of i.v. transferred BM monocytes may lead to the gradual reseeding of infected lung tissue with undifferentiated CD11b⁺ CD11c⁻ monocytes, which could explain the persistence of this cell population over time.

Compared with uninfected lung tissue, we observed that CD11c up-regulation by monocytes recruited to Mtb-infected lung and PLN tissue occurred more rapidly, and the amount of cell surface CD11c was significantly higher. The CD11b⁺ CD11c⁺ population in infected lungs was of particular interest because this is the subset that contains both iNOS⁺ effector cells, but also cells that we think can differentiate further into conventional DC. Classically activated Mϕ in Mtb lesions in the lungs are thought to be the main source of NO following infection, and NO production has long been regarded as a hallmark of Mϕ function, a notion that has been challenged in recent years with the discovery of iNOS expressing Tip-DC (TNF and iNOS producing DC) (34). Tip-DC were first identified in the spleens of mice infected with Listeria monocytogenes, and they share some features with the monocyte-derived iNOS⁺ cells in the lungs of Mtb-infected mice. Both subsets express CD11b, CD11c, and MAC-3. This raises the question of whether the iNOS⁺ cells we identified are Tip-DC? Despite the similarities, important differences exist. For example, while Tip-DC express Gr-1 and not CD205 or F4/80, the iNOS⁺ lung cells we identified in this study clearly expressed both CD205 and F4/80, but not Gr-1. Therefore, the iNOS⁺ cells in lungs of Mtb-infected mice do not seem to be typical Tip-DC.

CD11b⁺ CD11c⁺ cells in Mtb-infected or inflamed lungs have also been suggested to be DC (3, 15, 25). However, classifying these cells on the basis of expression of CD11b and CD11c expression is an oversimplification, since it is clear that CD11b⁺ CD11c⁺ cells are heterogeneous with respect to phenotype. Sorted CD11b⁺ CD11c⁺ cells from Mtb-infected lungs contain cells with a DC morphology but this subset also includes foamy Mϕ (3, 9). The lack of markers that can specifically discriminate DC from Mϕ is a significant problem. Alveolar Mϕ in the bronchoalveolar space of uninfected mice have a uniform CD11b⁺ CD11c⁻ phenotype; during acute inflammation, they up-regulate CD11b to become CD11b⁺CD11c⁺ (15, 28). Thus, some of the CD11b⁺ CD11c⁺ cells in the lungs of Mtb-infected mice could be activated alveolar Mϕ. In addition to lack of specificity of CD11c for DC, it is also not certain whether CD205 is a better lineage marker. Although CD205 is predominantly expressed by DC, it is also expressed by B cells and peritoneal macrophages (35–37). Importantly, inflammation may alter CD205 expression. While alveolar Mϕ from uninfected mice do not express CD205, foamy Mϕ in the pulmonary granulomas of Mtb-infected mice express CD205, and most CD205⁺ myeloid cells are CD11b⁺CD11c⁺ (9). Finally, induction of DC markers by Mϕ during their differentiation into foam cells occurs in vitro (38).

We think that the monocyte-derived iNOS⁺ cells we identified in Mtb-infected lungs are classically activated Mϕ. This is based on their large size and their cell surface expression of CD11b, MAC-3, and F4/80. Additionally, monocyte-derived Mϕ but not DC produce iNOS and NO after IFN-γ priming and LPS stimulation in vitro (data not shown). In an analogous manner, the in vivo differentiation of monocytes into cells with high cell surface expression of MAC-3 and iNOS production is IFN-γ-dependent. In fact, the expression of MAC-3 and CD205 appear to identify iNOS-producing activated Mϕ in the lungs of Mtb-infected mice. We have not identified where the adoptively transferred monocytes localize in the lung; however, since most iNOS expressing Mϕ are located in pulmonary granulomas, it is likely that transferred monocytes migrate into Mtb lesions where they differentiate into effector cells.

The adoptive transfer model provides a strategy to assess how perturbations of monocytes in an otherwise normal environment alter cell differentiation and acquisition of effector function. For example, we obtained BM monocytes from cytokine and chemokine receptor knockout mice to determine how these different signaling molecules affect monocyte differentiation. Thus, we found that based on CD11c and MHC class II expression, early monocyte differentiation to the CD11b⁺ CD11c⁻ stage was independent of several cytokine receptors, including IL-3R, IL-3Rβ/βR, and IFN-γR. While GM-CSF was sufficient to induce differentiation of monocytes into DC in vitro (data not shown), GM-CSF was not required for early monocyte differentiation in the inflamed lungs of Mtb-infected mice. Similarly, this process was independent of IL-3, IL-5, MCP-1, and IFN-γ.

We previously demonstrated that up-regulation of the Ag-presenting molecules CD1d and MHC class II on adoptively transferred inflammatory Mϕ recruited to Mtb-infected lungs is IFN-γ-dependent (23). Although of unknown origin, the IFN-γ-dependent MHC class II up-regulation by recruited Mϕ, along with IFN-γ-independent regulation of MHC class II cell surface expression by DC, in Mtb-infected lung tissue shown by Kincaid et al. is in agreement with our earlier results (39). However, the cells used in our previous study were TG-elicited peritoneal Mϕ, which are clearly more differentiated cells than the monocytes used in the present report. While class II MHC up-regulation on Mϕ requires IFN-γ activation, class II MHC expression by differentiating monocytes is IFN-γ-independent. Taken together, our results reveal a difference in
MHC class II regulation by differentiated Mφ and recruited monocytes in inflamed or infected tissues. Factors other than cytokines may be important for monocyte differentiation. Monocyte transmigration across endothelium may trigger differentiation and cell contact-dependent signaling, both with endothelial cells and other lung stromal cells, and may be critical particularly under inflammatory conditions (40, 41). From these data, we infer that there is considerable redundancy in the different signaling pathways that trigger differentiation of monocytes.

A similar approach was used to determine whether CCR2 was required for monocyte recruitment to the lung during pulmonary tuberculosis. We were particularly interested in this possibility since mice deficient in CCR2 expression have reduced numbers of Mφ and DC in the lungs following i.v. Mtb infection, and they are more susceptible to Mtb (2). CCR2 expression by transferred monocytes was not required for recruitment to Mtb-infected lung and PLN tissue. These results are consistent with the model that CCR2 is required for release of monocytes from the BM into circulation, and not for recruitment from blood vessels into infected tissue (11). Thus, by purifying BM monocytes and injecting them i.v., we may have bypassed the requirement for CCR2. However, with the exception of IFN-γR, we still do not know if monocyte differentiation into CD11b\textsuperscript{low}CD11c\textsuperscript{+} cells in Mtb-infected mice is dependent on the cytokine or chemokine receptors tested here.

An intriguing observation was the rather late appearance of conventional monocyte-derived DC in the lungs of infected mice. While iNOS production by Mφ was IFN-γ-dependent, monocyte differentiation into conventional DC was not. Temporally, the CD11b\textsuperscript{+}CD11c\textsuperscript{+} cells were detectable only after differentiation of CD11b\textsuperscript{+}CD11c\textsuperscript{+} cells; therefore, we propose that monocyte-derived CD11b\textsuperscript{+}CD11c\textsuperscript{+} cells include an intermediate subset that further differentiates into CD11b\textsuperscript{+}CD11c\textsuperscript{+} cells in vivo. Thus, monocyte-derived CD11b\textsuperscript{+}CD11c\textsuperscript{+} cells found in the lungs of Mtb-infected mice are a heterogeneous population that includes differentiated effector cells such as classically activated Mφ, as well as DC progenitors.

Monocytes constitutively exit peripheral tissue and enter draining lymph node via the afferent lymphatics. Herein we show that monocytes are recruited to the PLN draining the Mtb-infected lungs. In some respects, monocyte differentiation in the PLN was similar to monocyte differentiation in the infected lung tissue, including rapid up-regulation of high CD11c levels along with up-regulation of MHC class II and MAC-3. Also, similar to differentiating monocytes in the Mtb-infected lung, monocytes recruited to the inflamed PLN down-regulated CD11b. Still, monocyte differentiation in infected lungs compared with infected PLN was strikingly different. While monocytes recruited to the lung tissue gave rise to large (FSC\textsuperscript{high}) iNOS\textsuperscript{+} classically activated Mφ, monocytes recruited to the infected PLN did not differentiate into activated Mφ, but remained FSC\textsuperscript{low}iNOS\textsuperscript{−} and acquired a mature DC phenotype, including high cell surface expression of CD86 and CD83.

In this study we present data obtained using a newly developed monocyte adoptive transfer system, which supports a model in which monocyte differentiation and acquisition of effector function are dramatically altered by infection and inflammation. These processes are dependent on the type of inflammatory stimuli and tissue localization of the recruited monocytes. Our monocyte adoptive transfer model allowed us to directly demonstrate that monocytes recruited to the lung can differentiate into classically activated iNOS-producing Mφ during Mtb infection. Our results show that monocytes are progenitor cells for multiple Mφ and DC subsets in uninfected lung and in Mtb-infected lung and PLN tissue, providing the first direct evidence for monocyte differentiation into both Mφ and DC following infection with a live human pathogen. These findings provide insight into how recruited monocytes can influence multiple aspects of the host immune response during infectious diseases affecting the lung.

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Disclosures
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References


