Lung Neutrophils Facilitate Activation of Naive Antigen-Specific CD4+ T Cells during Mycobacterium tuberculosis Infection

Robert Blomgran and Joel D. Ernst

*J Immunol* 2011; 186:7110-7119; Prepublished online 9 May 2011;
doi: 10.4049/jimmunol.1100001
http://www.jimmunol.org/content/186/12/7110

References

This article cites 54 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/186/12/7110.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Lung Neutrophils Facilitate Activation of Naive Antigen-Specific CD4+ T Cells during Mycobacterium tuberculosis Infection

Robert Blomgran* and Joel D. Ernst*†‡

Initiation of the adaptive immune response to Mycobacterium tuberculosis occurs in the lung-draining mediastinal lymph node and requires transport of M. tuberculosis by migratory dendritic cells (DCs) to the local lymph node. The previously published observations that 1) neutrophils are a transiently prominent population of M. tuberculosis-infected cells in the lungs early in infection and 2) that the peak of infected neutrophils immediately precedes the peak of infected DCs in the lungs prompted us to characterize the role of neutrophils in the initiation of adaptive immune responses to M. tuberculosis. We found that, although depletion of neutrophils in vivo increased the frequency of M. tuberculosis-infected DCs in the lungs, it decreased trafficking of DCs to the mediastinal lymph node. This resulted in delayed activation (CD69 expression) and proliferation of naive M. tuberculosis Ag85B-specific CD4+ T cells in the mediastinal lymph node. To further characterize the role of neutrophils in DC migration, we used a Transwell chemotaxis system and found that DCs that were directly infected by M. tuberculosis migrated poorly in response to CCL19, an agonist for the chemokine receptor CCR7. In contrast, DCs that had acquired M. tuberculosis through uptake of infected neutrophils exhibited unimpaired migration. These results revealed a mechanism wherein neutrophils promote adaptive immune responses to M. tuberculosis by delivering M. tuberculosis to DCs in a form that makes DCs more effective initiators of naive CD4 T cell activation. These observations provide insight into a mechanism for neutrophils to facilitate initiation of adaptive immune responses in tuberculosis. The Journal of Immunology, 2011, 186: 7110–7119.

Despite the availability of drugs to treat it, tuberculosis (TB) remains a major burden to human health. Mycobacterium tuberculosis infects via inhalation and resides in diverse professional phagocytes in the lungs where it uses strategies, such as preventing phagosome maturation and subversion of host cell-death pathways, to survive and replicate (1). Effective immunity against M. tuberculosis requires CD4+ Th1 and CD8+ T lymphocyte responses to M. tuberculosis Ags (2–5). Compared with other lower respiratory tract infections, such as influenza A (6), for which the peak in naive T cell proliferation occurs 4 d postinfection, the onset of the CD4+ response against M. tuberculosis is delayed until 10–12 d after aerosol infection (7–9), giving the bacterium time to expand and establish a niche that allows it to resist eradication. Polymorphonuclear neutrophils are abundant, motile cells involved in the innate immune response and form an early line of defense against microbial pathogens. These professional phagocytes are crucial in the defense against extracellular bacterial and fungal infections. Although parasites, such as Leishmania, have evolved to exploit neutrophils to establish and promote disease (10), neutrophils play a protective role against certain intracellular pathogens (11–14). In an in vivo intranasal M. bovis bacille Calmette–Guérin (BCG) infection model, neutrophils were suggested to have a dual role in acute infection, a direct antimicrobial activity counterbalanced by anti-inflammatory properties (15). Furthermore, innate immune responses to M. tuberculosis in RAG-deficient mice revealed a compensatory function for neutrophils in keeping the bacterial burden in check in the absence of IFN-γ (16). In addition to a direct bactericidal or immunomodulatory effect, neutrophils readily undergo apoptosis, and phagocytosed microbe-containing apoptotic neutrophils can have a stimulatory effect on macrophages (17) and dendritic cells (DCs) (18). Additionally Davis and Ramakrishnan (19) clearly showed that spread of bacteria through apoptotic cells is a major mechanism by which macrophages obtain virulent mycobacteria in vivo. Although neutrophils were shown to contribute to innate protection against mycobacteria (15, 16, 20–23), data to the contrary are similarly compelling (15, 24–26). Other than the neutrophil’s capacity to produce chemokines/cytokines (27–30), in vivo evidence for a role of neutrophils in modulating adaptive immunity during M. tuberculosis infections has not been reported.

Evidence for one or more roles of neutrophils in human immunity to TB includes the observation that the risk for TB infection among household contacts is inversely associated with peripheral blood neutrophil count, and killing of M. bovis BCG in a whole-blood in vitro assay was significantly impaired by neutrophil depletion (20). Moreover, humans exhibit a transcriptional signature in peripheral blood that indicates a role for neutrophils and/or a related myeloid cell that occurs in response to active pulmonary TB (31). Consequently, a greater understanding of the roles that neutrophils play in the innate and adaptive immune responses to M. tuberculosis is needed.
DCs are potent APCs that prime naive T cells in the lung-draining lymph node (mediastinal lymph node [MDLN]) following *M. tuberculosis* infection (32, 33). Initial activation of naive *M. tuberculosis*-specific CD4+ T cells in the MDLN depends on DC transport of bacteria from the lungs to the MDLN (9), in an IL-12p40 homodimer- (32) and temporally CCR7-dependent manner (34). Furthermore, when characterizing the cells harboring *M. tuberculosis* following aerosol infection of mice, we found that neutrophils were a transiently dominant population of lung cells infected early in infection (35). The observation that the peak number of infected neutrophils immediately preceded the peak of infected DCs in the lungs suggests at least two competing hypotheses: acquisition of *M. tuberculosis* by neutrophils transiently sequesters the bacteria and delays their acquisition by DCs, or infected neutrophils interact with DCs to promote DC acquisition of the bacteria and bacterial Ags. To test these hypotheses and to characterize the role of neutrophils in the initiation of adaptive immune responses to *M. tuberculosis*, we depleted neutrophils in vivo using a mAb against the neutrophil-specific Ag Ly6G (clone 1A8) (15, 36). We found that neutrophils were necessary for timely initiation of the adaptive immune response by supporting DC migration and trafficking of *M. tuberculosis* to the local lymph node.

**Materials and Methods**

**Mice**

C57BL/6 mice were bred and housed in a specific pathogen-free environment in New York University School of Medicine animal facilities or purchased from The Jackson Laboratory (Bar Harbor, ME). P25TCR-Tg mice, whose CD4+ T cells express a transgenic T cell receptor that recognizes peptide 25 (aa 240–254) of *M. tuberculosis* Ag85B bound to H-2Kd, were on a C57BL/6 background (CD45.2) or on a Rag-1−/− background (when specified), as previously described (37), and were bred in the New York University School of Medicine animal facilities. CD45.1 mice were either bred in New York University School of Medicine animal facilities or purchased from Taconic Farms. Genotypes of mice were confirmed by PCR testing of tail genomic DNA. All procedures conducted on mice were in accordance with the conditions specified by the New York University School of Medicine Institutional Animal Care and Use Committee.

**Abs, FACS staining, and acquisition**

All Abs were purchased from BD Pharmingen, unless otherwise stated. Anti-CD11c PerCP (H3L) (1:200) was custom conjugated from BD Pharmingen, and other Ab conjugates used were anti-CD45.2 PerCP (1:200), anti-CD4 Alexa Fluor 647 (1:200), anti-CD68 allophycocyanin or PE (1:200), anti-CD80 Alexa Fluor 647 or PE (1:200), anti-MHC class II Alexa Fluor 488 or PE (1:100), CCR7 Alexa Fluor 647 or PE (1:200), anti-Ly6C FITC or PE (1:600), anti-Ly6G Alexa Fluor 647 or PE (1:100), and Gr-1 allophycocyanin or PE (1:200). Staining for surface markers was done by resuspending up to 1 × 10^6 cells in 100 µl FACS buffer (PBS supplemented with 1% heat-inactivated FBS, 0.1% NaCl, and 1 mM EDTA) containing Abs and incubating at 4°C for 30 min (or at 37°C for CCR7). Cells were washed twice and fixed overnight in PBS/1% paraformaldehyde at 4°C. Data were acquired using a FACSCalibur or LSR II flow cytometer, depending on the experiment.

**P25TCR-Tg CD4+ T cell isolation and labeling**

P25TCR-Tg mice, between 8–16 wk of age, were killed according to approved laboratory animal procedures, and naïve P25TCR-Tg CD4+ T cells from lymph nodes and spleen were isolated, as previously described (9). For proliferation assays, CD4+ T cells were labeled with CFSE (Invitrogen).

**Adoptive transfer and aerosol infection**

CD4.1 mice routinely received 2–3 × 10^6 CFSE-labeled P25TCR-Tg CD4+ T cells (CD45.2), by tail vein or retro-orbital injection, in 100 µl sterile PBS. Three to twenty-four hours postcell transfer, mice were infected by the aerosol route using an Inhalation Exposure Unit (Glas-Col), The infectious dose was confirmed by euthanizing four or five mice and plating homogenized lungs within 24 h of infection, as previously described (35).

**Tissue processing and CFU determination**

Mice were euthanized at designated time points, and tissues were used to prepare single-cell suspensions and to determine the bacterial loads by plating, as previously described (9, 35).

**Phenotyping and quantitation of lung cells**

To avoid epitope masking in mice treated with the neutrophil-depleting Ab to Ly6G, 1A8, neutrophils were defined and quantitated as CD11b^hi/Gr-1^hi/CD45.1^~^ (35, 36, 38). For identification of lung macrophage and DC subsets, neutrophils were first gated out. Based on previous functional and morphological characterization, lung cell subsets were designated as alveolar macrophages (CD11b^int/CD11c^lo/CD86^lo^), myeloid DCs (mDCs; CD11b^hi/CD11c^lo/CD86^hi^), recruited macrophages (CD11b^lo/CD11c^int/CD86^lo^), or monocytes (CD11b^hi/CD11c^int/CD86^hi^) (35, 39).

**In vivo neutrophil depletion**

The purified Ly6G-specific Ab 1A8 (36) was used to deplete neutrophils in vivo, and purified 2A3 (Rat IgG2a) was used as isotype control Ab; both were obtained from BioXcell (West Lebanon, NH). Single-dose treatment (300 µg administered i.p.) was used to prevent the confounding effects of an immune response toward the depleting Ab, which can be seen with multiple treatments in vivo (25). Furthermore, 1A8 had no effect on Ly6C+ cells in spleen or lungs 2 d after administration, at which time neutrophils were fully depleted (data not shown) (12).

**Bacterial strains, treatment, and in vitro infection**

*M. tuberculosis* strain H37Rv and FACS-optimized GFP-H37Rv (under the control of the Mycobacterium bovis BCG Hsp60 promoter) were prepared for use in vivo and in vitro use, as previously described (35). For in vivo use, log-phase bacteria (OD_{560} = 0.5–0.9) were washed, resuspended in 15 mg/ml mouse GM-CSF–supplemented RPMI 10 complete medium (referred to as GM-CSF medium) and gravity filtered through a 5-µm filter to obtain single-cell bacteria. Multiplicity of infection (MOI) was calculated, depending on the OD at 580 nm, and validated through serial dilutions and plating. DCs were infected at MOI = 5 for 19 h, resulting in infection of 60–70% of bone marrow DCs (BMDCs). To optimize uptake of *M. tuberculosis* by neutrophils and synchronize the assay, log-phase GFP-H37Rv bacteria were opsonized using 50% pooled AB human serum in RPMI 1640 without additives for 30 min at 37°C, before addition to neutrophils (MOI = 5), allowing for 40 min of phagocytosis at 37°C (routinely yielding 70–80% GFP+ neutrophils, according to flow cytometry). Infected cells were washed three times, treated for 40 min with 200 µg/ml amikacin, and washed twice more before being used. Neutrophils were additionally labeled with CMTMR (CellTracker Orange; Invitrogen) for selected experiments.

**BMDCs and neutrophils**

Bone marrow from C57BL/6 mice was cultured in GM-CSF medium at 37°C, 5% CO_2. Fresh GM-CSF medium was added on days 3 and 6. The floating cell fraction was collected at day 7 and used as source of BMDCs and neutrophils by positive selection, using magnetic beads coupled to anti-CD11c mAb (NA18) and anti-Ly6-6G, respectively, and sorted by AutoMACS, according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Cells were maintained in GM-CSF medium throughout the experiment to prevent cytokine withdrawal-induced cell death. GM-CSF medium is RPMI 1640 with 10% heat-inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 1% β-mercaptoethanol, 10 mM HEPES, and 15 ng/ml mouse GM-CSF; culture supernatants from GM-CSF–producing melanoma cells were quantified using a mouse GM-CSF ELISA kit (BioSource International) and stored as aliquots at −80°C until use.

**Fli3 ligand-expanded in vivo DCs**

To increase the number of in vivo DCs, C57BL/6 mice were injected s.c. with 3–6 × 10^6 Fli3 ligand (Fli3L)-producing melanoma cells at 80% confluence, and splenocytes were isolated 7–9 d later. Splenocytes were excised and forced through a 70-µm nylon cell strainer (BD Falcon), and RBCs were removed using ACK lysis buffer. These in vivo-derived DCs (mDCs; CD11b^hi/CD11c^lo/CD86^hi^) were fully depleted (data not shown) (12).
contrast, immature DCs were CD40<sup>neg</sup>, CD80<sup>neg</sup>, CD86<sup>neg</sup>, and MHCI class II<sup>−</sup> Maturation of DCs was further validated using fluid-phase endocytosis of FITC-dextran; mature DCs routinely showed 50–75% decreased endocytosis compared with immature DCs. Cellular uptake of FITC-dextran (m.w. 40,000; Sigma, St. Louis, MO) was quantified by flow cytometry and expressed as mean fluorescent intensity, from which the background fluorescence of cells incubated with FITC-dextran at 4°C was subtracted.

**Generation of P25 Rag1<sup>−/−</sup> effector T cells**

P25TCR-Tg CD4<sup>+</sup> T cells on a Rag1<sup>−/−</sup> background were cultured with irradiated splenic APCs in the presence of 0.5 μM peptide 25, 10 ng/ml IL-12p70, 5 ng/ml IL-2, and 50 ng/ml anti-IL-4 for 3 d. T cells were further cultured in fresh media containing only IL-2 without peptide 25 for the next 4 d. At the time of assay, cells were washed and added to DCs in GM-CSF medium without IL-2.

**In vitro T cell-stimulation assay**

BMDCs were plated at 10<sup>5</sup> cells/well in flat-bottom 48-well tissue culture plates and allowed to adhere for 4 h. Thereafter, DCs were infected with M. tuberculosis or stimulated with M. tuberculosis-containing/aminacinketone-treated neutrophils (1:1 DCs/neutrophils). After 19 h of incubation, potential extracellular bacteria were removed with washing with PBS. P25TCR-Tg Rag1<sup>−/−</sup> effector T cells or CFSE-labeled naive P25TCR-Tg CD4<sup>+</sup> T cells were added at 4 × 10<sup>5</sup> cells/well in GM-CSF medium (1 ml total volume). For analyzing activation of effector T cells, supernatants were collected and sterile filtered (0.22 μm) before assay for IFN-γ by ELISA (BD Bioscience). Analysis for proliferation of naive P25TCR-Tg CD4<sup>+</sup> T cells was done by evaluating CFSE dilution by flow cytometry (9, 34).

**In vitro migration assay**

DCs (1.5 × 10<sup>5</sup>) were added to upper wells of 5-μm-pore size polycarbonate filters in 6.5-mm diameter 24-well Transwell chambers (Corning via Fisher) in 100 μl with 600 μl chemokine or medium alone (to determine spontaneous migration) in the lower wells. Recombinant mouse MIP-3β/CCL19 was added to a final concentration of 2 nM (R&D Systems). Migration assays were conducted in GM-CSF medium for 1 h (FLt3L-derived in vivo DCs) or 2–3 h (BMDCs); longer times increased the spontaneous migration without affecting directed migration. Thereafter, 60 μl 30 mM EDTA was added to lower wells and incubated for 5 min at 37°C. The migrated cells collected from the lower wells were stained with Abs and fixed overnight in PBS/1% paraformaldehyde. Migrated cells and the number of starting cells were counted for 1 min at a constant flow rate, with 600 μl chemokine or medium alone. Background fluorescence of cells incubated with FITC-dextran at 4°C was background fluorescence of cells incubated with FITC-dextran at 4°C was subtracted. Background fluorescence of cells incubated with FITC-dextran at 4°C was subtracted. Background fluorescence of cells incubated with FITC-dextran at 4°C was subtracted.

**Statistical analysis**

Unless otherwise indicated, statistical comparison was performed by the unpaired, two-tailed Student t test, using Prism 4 for Macintosh (version 4.0a, GraphPad, San Diego, CA). The p values < 0.05 were considered significant, but p < 0.01 and p < 0.001 are also shown when appropriate.

**Results**

**I8A efficiently depletes neutrophils in M. tuberculosis-infected mice**

We initiated our studies of the role of neutrophils in immune responses to M. tuberculosis by characterizing the kinetics of neutrophil accumulation in the bronchoalveolar lavage fluid (BALF) and lung parenchyma after low-dose aerosol infection. We found a small population of neutrophils (1.5–1.7 × 10<sup>5</sup> neutrophils/mouse) in the parenchyma on days 1 and 4 postinfection. Beginning on day 7, there was a progressive increase in the number of neutrophils in the lung for the duration of the experiments (Fig. 1A). Because the number of neutrophils in the BALF was <1% of the total lung neutrophils at any time point studied, we did not separate BALF and lung parenchymal neutrophils in subsequent experiments. To determine whether neutrophils promote or deter T cell activation during initiation of the adaptive immune response to M. tuberculosis, we depleted neutrophils in C57BL/6 mice 9 d postinfection, before the earliest activation of CD4<sup>+</sup> T cells is observed (3, 8, 9, 34). First, using the conventional Gr-1<sup>high</sup>/CD11b<sup>high</sup> gating strategy for neutrophils, which was confirmed by Gr-1/Ly6C<sup>−</sup> staining (38) (Fig. 1B), we established that a single dose of I8A depleted neutrophils (Gr-1<sup>high</sup>, CD11b<sup>high</sup> from blood (by 94.3 ± 3.12%) and lungs (by 89.6 ± 2.76%) in infected mice (mean depletion ± SD; n = 3; flow plots for lungs shown in Fig. 1B). Furthermore, we verified that I8A showed no epitope masking that would prevent subsequent detection of residual neutrophils by the widely used Ab to Gr-1 (clone RB6-8C5, which recognizes both Ly6G and Ly6C) (data not shown). Next, we determined that administration of I8A on day 9 postinfection caused profound depletion of neutrophils in the lungs until ≈day 14 and that neutrophils remained significantly depleted through day 21 (Fig. 1E). No other cell subset examined was depleted. However, depletion of neutrophils caused a small, but significant, increase in the number of mDCs, recruited macrophages, and monocytes in the lungs compared with isotype-treated control mice (Fig. 1E), confirming that neutrophils do not contribute to killing of M. tuberculosis during this phase of the infection. This is in accord with the study by Pedrosa et al. (21), who showed that administration of a Ly6G/Ly6C-specific mAb (RB6-8C5) at day 16 postinfection with M. tuberculosis, via i.v. infection, did not affect the total bacterial burden in liver, spleen, or lungs of BALB/c mice.

**Neutrophil depletion results in cellular redistribution of M. tuberculosis in the lungs**

To determine whether neutrophils delay or accelerate the acquisition of M. tuberculosis by mDCs in the lungs, mice were treated with I8A or isotype control at day 16 postinfection and analyzed at day 19. This time point was chosen because it overlaps with the peak of infected neutrophils in the lungs of untreated mice (35) and because few mDCs are present in the lungs at earlier time points. We used M. tuberculosis expressing FACS-optimized GFP to identify the infected cells by flow cytometry. With depletion of neutrophils, there was a 2-fold increase in the total number of infected DCs (Fig. 2A). This redistribution of M. tuberculosis was not specific to DCs, because recruited macrophages and monocytes showed similar increases, whereas alveolar macrophages did not. Furthermore, when considering all of the infected lung cell subsets, ≥98% of the GFP<sup>+</sup> events in the control group were accounted for in the I8A-treated group. Quantitation of M. tuberculosis CFU in tissue homogenates revealed no difference in bacterial loads in lungs of neutrophil-depleted versus isotype-treated control mice when treated at day 16 and analyzed at day 21 postinfection (Fig. 2B).

**Neutrophils promote timely proliferation of M. tuberculosis Ag85B-specific CD4<sup>+</sup> T cells**

We previously reported that proliferation of naive P25TCR-Tg CD4<sup>+</sup> T cells occurs in the MDLN (and not the lungs) and that this initiation of the adaptive immune response to M. tuberculosis Ag85B requires transport of live bacteria from the lungs to the local draining lymph node (9). To characterize the timing and dependence on neutrophils in activation of naive M. tuberculosis-specific T cells, we adoptively transferred CFSE-labeled P25TCR-Tg CD4<sup>+</sup> T (CD45.2) cells into C57BL/6 mice (CD45.1) prior to infection with M. tuberculosis. Mice were treated with I8A or isotype control at day 9, and P25TCR-Tg T cells in the lung...
draining lymph node were analyzed for CD69 expression and proliferation at days 14, 15, and 17 postinfection. No evidence of T cell proliferation was detected in either group at day 14; however, there was increased expression of the activation marker CD69 in the isotype- versus the 1A8-treated group (40.2 ± 5.2% and 20.7 ± 3.5% of adoptively transferred cells, respectively; p = 0.0223) (top left panel, Fig. 3A; Fig. 3B), indicating earlier activation of lymph node CD4+ T cells in the presence of lung neutrophils. At day 15, isotype-treated mice showed a significant increase in P25TCR-Tg CD4+ T cells that had undergone at least one cycle of proliferation compared with those in neutrophil-depleted mice (top right panel, Fig. 3A; Fig. 3C). By day 17 postinfection, active proliferation of CD4+ T cells was observed in neutrophil-depleted mice, and there was no difference in CD4+ T cell proliferation between the groups, indicating that neutrophil depletion delays rather than prevents M. tuberculosis-specific T cell responses.

Neutrophils promote trafficking of M. tuberculosis from lungs to the MDLN

Although neutrophils were essential for timely activation of naive T cells in the MDLN, we did not observe neutrophils (Gr-1high/CD11bhigh) in the lymph node at any time point studied, suggesting that the effects of neutrophils that promote naive T cell activation occur in the lungs. Because we (9) and other investigators (7) found that activation of M. tuberculosis-specific T cells is preceded by transport of live bacteria to the lymph node, we examined the effect of neutrophil depletion on trafficking of bacteria to the lymph node. This revealed ~2-fold more M. tuberculosis CFU in lymph nodes of neutrophil-replete mice compared with neutrophil-depleted mice at days 14 and 15, respectively (Fig. 4A), in the absence of a difference in lung CFU at any time point examined (Fig. 1D). We previously found that 1200–1500 M. tuberculosis CFU seemed to represent a threshold for promoting proliferation of P25TCR-Tg CD4+ T cells in the MDLN (9). The present data (Figs. 3C, 4A) are consistent with this, and indicated that neutrophil-replete mice reach this threshold sooner than do neutrophil-depleted mice (Fig. 4A). Finally, despite the greater number of mDCs in lungs of neutrophil-depleted animals (Fig. 1E), trafficking of DCs to the MDLN was actually delayed by neutrophil depletion (Fig. 4B). In ac-
cordance with the presence of fewer bacteria and DCs in the lymph node in the absence of neutrophils, it was not surprising to find that the number of \textit{M. tuberculosis}-infected (GFP +) cells among the mDC (CD11b high/CD11chigh) population in the MDLN was also decreased on day 15 in the neutrophil-depleted group (941.2 ± 226 versus 480.1 ± 87.47 in neutrophil-replete and neutrophil-depleted mice, respectively; \( p = 0.0468; \ n = 5 \)). In contrast to the reduced number of CD11b high/CD11chigh mDCs in the MDLN, the number of CD11b low and CD11b neg DCs in the MDLN were unaffected by neutrophil depletion with 1A8 at any time point studied (data not shown). Together, these data indicated that neutrophils are important for timely acquisition and trafficking of DCs containing \textit{M. tuberculosis} from the lungs to the MDLN.

FIGURE 3. Neutrophils accelerate activation of naive \textit{M. tuberculosis} Ag85B-specific CD4 + T cells in vivo. Mice were treated as shown in Fig. 1C after adoptive transfer of naive P25TCR-Tg CD4 + T cells on the day preceding infection. \( A \), Representative CFSE-dilution profiles of P25TCR-Tg CD4 + T cells in MDLN on days 14, 15, and 17 postinfection. \( B \), P25TCR-Tg cell activation as reflected by CD69 expression; gating used is shown in A. \( C \), Comparison of P25TCR-Tg CD4 + T cell proliferation in neutrophil-depleted and control mice; gating used is shown in A. Data in \( B \) and \( C \) are mean ± SD of five mice per group and time point. Data are representative of two separate experiments. **\( p < 0.01 \).

FIGURE 4. Neutrophils accelerate trafficking of \textit{M. tuberculosis} and DCs from the lungs to the MDLN. Mice were treated as shown in Fig. 1C. \( A \), \textit{M. tuberculosis} CFU in MDLN homogenates at the indicated time points. \( B \), CD11b high/CD11c high mDCs on days 14 and 15 postinfection. Data are mean ± SD of five mice per group and time point, representing data from two (A) or one (B) separate experiments. Data in \( B \) were analyzed using the unpaired one-tailed Student \( t \) test. *\( p < 0.05 \).
M. tuberculosis-infected neutrophils efficiently provide Ags to DCs for activation of CD4+ T cells

Because the peak population of M. tuberculosis-infected neutrophils precedes that of infected DCs in the lungs (35), and because depletion of lung neutrophils delayed activation of CD4+ T cells in the lymph node, we considered the possibility that M. tuberculosis-infected neutrophils interact with DCs in the lungs in a manner that promotes subsequent activation of naive CD4+ T cells. We first examined the possibility that infected neutrophils can provide M. tuberculosis Ags to DCs for presentation to CD4+ T cells in vitro. To compare the ability of DCs to present M. tuberculosis Ag85B after direct infection or after the bacteria had first been ingested by neutrophils, we provided BMDCs with M. tuberculosis directly or with the same number of bacteria contained in neutrophils and assayed the activation of P25TCR-Tg CD4+ effector T cells. In particular, we quantitated effector T cell activation as IFN-γ secretion after 6, 12, 24, and 36 h of coinoculation. This revealed that activation of Ag-specific CD4+ T cells was as efficient when DCs were provided bacteria contained in neutrophils as when the DCs acquired bacteria directly (Fig. 5A). These data indicated that DC uptake of M. tuberculosis-infected neutrophils results in efficient presentation of bacterial Ags. To further characterize the presentation of M. tuberculosis Ags after neutrophil infection, we determined whether the ability of DCs to acquire and present Ags from infected neutrophils also occurred at lower MOIs. This revealed that DCs that acquired M. tuberculosis Ag from infected neutrophils activated CD4+ T cells as effectively as did directly infected DCs at all MOIs examined (Fig. 5B). Next, we examined the ability of DCs to stimulate proliferation of naive Ag85B-specific CD4+ T cells after incubation with infected neutrophils or with M. tuberculosis alone. This revealed that DCs that contained bacteria acquired from infected neutrophils were as efficient at inducing proliferation of naive P25TCR-Tg cells as were DCs that contained bacteria acquired by direct infection (Fig. 5C). These data indicated that Ag presentation by DCs is as efficacious whether M. tuberculosis Ags are acquired from infected neutrophils or obtained by direct infection.

Acquisition of bacteria from infected neutrophils prevents M. tuberculosis inhibition of DC migration

Because we observed that depletion of neutrophils delayed DC migration and trafficking of M. tuberculosis from the lungs to the MDLN, we hypothesized that neutrophils promote migration of DCs in the context of M. tuberculosis infection. Such a hypothetical effect could operate by directly promoting migration or by abrogating a negative effect of M. tuberculosis on migration. Therefore, we determined whether acquiring bacteria through distinct routes (either directly or by ingesting infected neutrophils) affects the migratory properties of DCs in response to CCL19, a chemoattractant for mature DCs. To compare migration of infected and uninfected DCs from a mixed population, we used GFP-expressing M. tuberculosis and flow cytometry to distinguish infected and uninfected cells in the migration assay. We also used

![FIGURE 5](http://www.jimmunol.org/)  

*FIGURE 5.* M. tuberculosis-infected neutrophils provide Ag to DCs for presentation to CD4+ T cells. A, BMDCs were directly infected with *M. tuberculosis* (Mtb; MOI = 5) or incubated with an equal number of neutrophils that were previously infected with *M. tuberculosis* (MOI = 5) for 19 h. Thereafter, BMDCs were incubated with P25TCR-Tg Th1 effector CD4+ T cells, and release of IFN-γ into supernatants was analyzed after 6, 12, 24, or 36 h. B, BMDCs were directly infected with *M. tuberculosis* at the MOI shown or incubated with an equal number of *M. tuberculosis*-infected neutrophils (previously infected at the MOI shown) and then incubated with P25TCR-Tg Th1 effector CD4+ T cells. Release of IFN-γ into supernatants was analyzed after 12 h. C, CFSE-labeled naive P25TCR-Tg CD4+ T cells were added to BMDCs that were either directly infected or after incubation with infected neutrophils, as described for A and B. T cell proliferation was analyzed at 72 h. Mean values of cells that had undergone at least one cycle of proliferation are depicted in graphs. Data in A–C are representative of two separate experiments performed in triplicate. Un, undetected.
CMTMR-labeled neutrophils to identify DCs that had ingested neutrophils (Fig. 6A). The upper panel of Fig. 6A shows the gating strategy excluding extracellular neutrophils (Ly6G−), and R1–R4 in Fig. 6B indicate the quadrant of the lower panel in Fig. 6A that was analyzed for migration. Approximately 20% of the uninfected control cells, as well as the DCs containing infected neutrophils (R2; Ly6G−/CD11c+/GFP+/CMTMR−), migrated in response to CCL19, whereas ~7% of the DCs directly infected with M. tuberculosis (R4; CD11c−/GFP−) migrated to the lower chamber during the 3-h assay. The decreased migration of directly infected DCs was associated with decreased expression of the chemokine receptor CCR7 compared with that expressed by DCs that contained M. tuberculosis-infected neutrophils (Fig. 6C). This indicated that direct M. tuberculosis infection of DCs causes a 2.4-fold decrease in migration compared with that of uninfected cells and that this inhibitory effect of M. tuberculosis is absent when DCs acquire M. tuberculosis by ingesting infected neutrophils. These data indicated that although Ag presentation and activation are equivalent between the groups, DCs that acquired M. tuberculosis through neutrophils are superior at migrating toward lymph node chemokines compared with directly infected DCs.

We also examined the effect of M. tuberculosis on migration of in vivo-derived CD11b+/CD11c+ spleen DCs. Flt3L-expanded in vivo DCs were more mature than were BMDCs, making them ideal for migration studies. However, as one manifestation of their mature phenotype, these cells did not ingest neutrophils, thereby restricting our study to examining the effects of direct infection with M. tuberculosis on migration. As we observed with BMDCs, a lower fraction of in vivo-generated DCs migrated in response to CCL19 if they contained M. tuberculosis (GFP− DCs) than if they did not contain bacteria (Fig. 6D). As we found with BMDCs, direct infection of Flt3L-expanded DCs by M. tuberculosis decreased expression of CCR7 (data not shown). Of note, the GFP− (noninfected) DCs from the M. tuberculosis-treated sample exhibited a 2.4-fold increase in migration compared with GFP+ DCs (p = 0.0037), as well as a 1.4-fold increase in migration compared with DCs that were not exposed to M. tuberculosis (p = 0.0425).

M. tuberculosis-infected neutrophils release a chemoattractant for DCs

To further investigate the interaction of neutrophils and DCs, we tested the hypothesis that M. tuberculosis-infected neutrophils release factors that attract DCs to facilitate the interactions of these cells in the lungs. We examined culture supernatants from uninfected and M. tuberculosis-infected neutrophils for their capacity to attract previously untreated BMDCs. Postinfection, neutrophils were extensively washed, treated with amikacin, and incubated for 19 h in fresh media. Cleared supernatants were filtered through a 0.22-μm filter before use in the lower wells of the

**FIGURE 6.** M. tuberculosis inhibits DC migration when DCs acquire bacteria directly but not when they acquire bacteria by ingesting infected neutrophils. Before migration assays, DCs were directly infected with GFP-M. tuberculosis (MOI = 5) or incubated with an equal number of either uninfected or infected (MOI = 5) CMTMR-labeled neutrophils for 19 h. To distinguish migration of DCs that contained bacteria, neutrophils, both, or neither, input cells and cells in the lower well of the Transwell chamber after migration were stained with Abs to CD11c and Ly6G, counted, and analyzed by flow cytometry. A, Gating strategy used to exclude extracellular neutrophils (Ly6G−, upper panels), and R1–R4 (lower panels) distinguish DCs that contain M. tuberculosis (GFP−), infected neutrophils (CMTMR+GFP+), or uninfected neutrophils (CMTMR−GFP−) (BMDCs used as input cells are displayed). B, BMDCs were allowed to migrate for 3 h in the presence of CCL19. C, CCR7 expression on uninfected DCs, directly infected DCs, and DCs that acquired M. tuberculosis by ingesting infected neutrophils. D, In vivo-expanded spleen DCs (Flt3L-DC) did not ingest neutrophils but could be infected by M. tuberculosis, restricting the evaluation of Flt3L-DC migration to cells incubated with M. tuberculosis (containing bacteria or not) and untreated DCs. In addition to GFP+ DCs, GFP− DCs from the M. tuberculosis-stimulated group were analyzed. Unstimulated CCR7−/− Flt3L-DCs were used to determine baseline random migration. Data are expressed as mean percentage (± SD) of the input cells that migrated to the lower chamber after 1 h. Each condition was assayed in triplicate; data are representative of two (B, C) and three (D) independent experiments. *p < 0.05, **p < 0.01. infPMN, M. tuberculosis-infected neutrophils; Mtb, M. tuberculosis; uninPMN, uninfected neutrophils.
Transwell migration system. Medium from uninfected neutrophils did not enhance DC migration above the level of spontaneous migration (with medium alone), whereas supernatants from infected neutrophils increased DC migration to levels comparable to 2 nM CCL19 (Fig. 7). When we used CCR7−/− BMDCs in the assay, there was no detectable difference in migration toward supernatant from uninfected or infected neutrophils (data not shown), indicating that the chemotactic activity produced by M. tuberculosis-infected neutrophils is attributable to one or more CCR7 agonists, either CCL19 or one of the isoforms of CCL21. This is consistent with previous evidence showing that human neutrophils release MIP-3β (CCL19) when stimulated with TNF-α, LPS, or Gram-positive or Gram-negative bacteria (40, 41).

**Discussion**

The findings reported in this article indicated that neutrophils contribute to activation of Ag-specific CD4+ T cells in response to M. tuberculosis in vivo. Using the Ly6G-specific Ab 1A8 to selectively deplete neutrophils, we found that neutrophils are necessary for optimal trafficking of M. tuberculosis from the lungs to the MDLN, promoting activation and proliferation of M. tuberculosis-specific CD4+ T cells. We further found that DCs that acquired M. tuberculosis through ingestion of neutrophils that contain bacteria are superior in migrating toward lymph node chemokines compared with directly infected DCs. Because we could not detect neutrophils (Gr-1high/CD11bhigh) in the MDLN at any time point studied, our data are most consistent with a model in which infected lung neutrophils convey M. tuberculosis to migratory DCs in a manner that bypasses inhibitory mechanisms of M. tuberculosis, ultimately supporting trafficking of M. tuberculosis-containing DCs to the MDLN and subsequent T cell activation. Our in vivo results confirm and extend prior in vitro observations that M. bovis BCG-infected neutrophils cooperate with DCs to result in DC maturation and effective Ag presentation to CD4 and CD8 T cells (42).

Several recent studies provided considerable insight into the process and mechanisms of activation of naive Ag-specific T lymphocytes postinfection with M. tuberculosis. These studies revealed that T cell activation occurs earliest in the lymph node downstream of the lungs, that T cell activation follows the appearance of live M. tuberculosis in the lymph node, and that live bacteria do not reach the lymph node until 8–10 d following aerosol infection (7, 9, 35). Moreover, recent studies established that mDCs are responsible for transporting M. tuberculosis from the lungs to the lymph node (32, 35), indicating that these cells play a key role in initiating adaptive immune responses to M. tuberculosis after aerosol infection. The data reported in this article, that neutrophils promote activation of Ag-specific CD4 T cells in TB by facilitating DC migration and Ag presentation, provide additional insight into the cellular events that contribute to the development of protective immunity in TB.

Several recent studies implicated neutrophils in contributing to immune responses in human TB. In particular, a study of contacts of active TB cases revealed that the risk for TB infection in contacts was inversely proportional to peripheral blood neutrophil counts, and neutrophils contributed to the killing of M. bovis BCG in a whole-blood assay (20). Our results in mice suggested that, in addition to a potential direct role in limiting infection, neutrophils contribute to development of an efficacious adaptive immune response. More recently, studies of gene expression in peripheral blood cells of subjects with active TB and a subset of those with latent TB infection revealed prominent expression of myeloid genes, especially neutrophils, further indicating a role for these cells in response to M. tuberculosis infection (31). Finally, ex vivo examination of samples of human bronchial lavage and tuberculous cavity contents revealed the association of a high proportion of acid-fast-stained M. tuberculosis with neutrophils in advanced stages of infection (43).

Historically, neutrophil-depletion studies in M. tuberculosis-infected animals focused on whether neutrophils have a bactericidal effect. For example, early (i.e., during the first week of infection) administration of the neutrophil-depleting Ab RB6-8C5 (which recognizes both Ly6G on neutrophils and Ly6C on certain mononuclear cells) to mice infected i.v. with a high dose of M. tuberculosis resulted in higher bacterial burdens in liver, spleen, and lungs, whereas late administration of RB6 had no effect on bacterial burden in any organ (21). Neutrophils may also contribute to local cytokine production (27–30), cellular recruitment and granuloma formation (44, 45), and optimal control of virulent Mycobacterium marinum infection in zebrafish embryos (46).

Although, the role of neutrophils in early activation of M. tuberculosis-specific T cells in vivo has not, to our knowledge, been studied previously, involvement of neutrophils in pathogen-specific adaptive immune responses was found for other intracellular pathogens. In the case of Leishmania, neutrophil deple- tion attenuates infection after transmission by sandflies (10) and promotes the development of parasite-specific immune responses in mice vaccinated with heat-killed Leishmania major Ag (47). Therefore, Leishmania exploits neutrophils to promote infection and to limit the efficacy of adaptive immune responses. In contrast, Listeria monocytogenes-infected neutrophils were found to serve as substrates for DC cross-presentation of both secreted and nonsecreted Ags in vitro, as well as for in vivo cross-priming of CD8+ T cells. Neutrophil depletion also decreased CD8+ T cell responses against nonsecreted L. monocytogenes Ags in vivo (48). In Salmonella-infected CCR6-deficient mice, adoptive transfer of wild-type blood neutrophils (Gr1−/CD11b+) restored T cell expansion and cell division of CFSE-labeled CD4+ T cells in the draining lymph node (49). Therefore, as in our studies, neutrophils may also contribute to the timely and optimal development of adaptive immune responses and, thereby, contribute to host protection against other intracellular pathogens.

In summary, we found that in mice infected with virulent M. tuberculosis by the aerosol route, neutrophils contribute to initial activation of Ag-specific CD4 T cells by cooperating with DCs in...
the lungs. Our results suggested that DCs acquire *M. tuberculosis* Ags by ingesting apoptotic neutrophils, indicating that inhibition of neutrophil apoptosis may contribute to the virulence of *M. tuberculosis* (50–54). Future studies of the roles of neutrophils in defense against *M. tuberculosis* are likely to reveal additional functions of these cells, which have traditionally been considered to function in innate immunity, but whose roles in modulating adaptive immune responses to intracellular pathogens are becoming more evident.

**Acknowledgments**

We thank Dr. Adrian Erlebacher and Mary Collins for CCR7–/– mice. We also thank Tawania Fergus for excellent technical assistance and Sofia Olmos and Smrita Srivastava for advice and assistance during early stages of the project.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


10. Fulfon, S. A., S. M. Reba, T. D. Martin, and W. H. Boon. 2002. Neutrophil-mediated mycobacterial cell death in the lungs. Our results suggested that DCs acquire *M. tuberculosis* Ags by ingesting apoptotic neutrophils, indicating that inhibition of neutrophil apoptosis may contribute to the virulence of *M. tuberculosis* (50–54). Future studies of the roles of neutrophils in defense against *M. tuberculosis* are likely to reveal additional functions of these cells, which have traditionally been considered to function in innate immunity, but whose roles in modulating adaptive immune responses to intracellular pathogens are becoming more evident.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


10. Fulfon, S. A., S. M. Reba, T. D. Martin, and W. H. Boon. 2002. Neutrophil-mediated mycobacterial cell death in the lungs. Our results suggested that DCs acquire *M. tuberculosis* Ags by ingesting apoptotic neutrophils, indicating that inhibition of neutrophil apoptosis may contribute to the virulence of *M. tuberculosis* (50–54). Future studies of the roles of neutrophils in defense against *M. tuberculosis* are likely to reveal additional functions of these cells, which have traditionally been considered to function in innate immunity, but whose roles in modulating adaptive immune responses to intracellular pathogens are becoming more evident.

**Acknowledgments**

We thank Dr. Adrian Erlebacher and Mary Collins for CCR7–/– mice. We also thank Tawania Fergus for excellent technical assistance and Sofia Olmos and Smrita Srivastava for advice and assistance during early stages of the project.


