Modulation of Naive CD4⁺ T-Cell Responses to an Airway Antigen during Pulmonary Mycobacterial Infection▼

Mursalin M. Anis, 1,2 Scott A. Fulton, 2 Scott M. Reba, 2 Clifford V. Harding, 1,‡ and W. Henry Boom 2,3†,*

Department of Pathology, 1 Division of Infectious Diseases, 2 and Tuberculosis Research Unit, 3 Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio

Received 25 October 2006/Returned for modification 28 November 2006/Accepted 2 February 2007

During pulmonary mycobacterial infection, there is increased trafficking of dendritic cells from the lungs to the draining lymph nodes. We hypothesized that ongoing mycobacterial infection would modulate recruitment and activation of antigen-specific naive CD4⁺ T cells after airway antigen challenge. BALB/c mice were infected by aerosol with Mycobacterium bovis BCG. At peak bacterial burden in the lungs (4 to 6 weeks postinfection), carboxy-fluorescein diacetate succinimidyl ester-labeled naive ovalbumin-specific DO11.10 T cells were adaptively transferred into infected and uninfected mice. Recipient mice were challenged intranasally with soluble ovalbumin (OVA), and OVA-specific T-cell responses were measured in the lungs, draining mediastinal lymph nodes (MLN), and spleens. OVA challenge resulted in increased activation and proliferation of OVA-specific T cells in the draining MLN of both infected and uninfected mice. However, only BCG-infected mice had prominent OVA-specific T-cell activation, proliferation, and Th1 differentiation in the lungs. BCG infection caused greater distribution of airway OVA to pulmonary dendritic cells and enhanced presentation of OVA peptide by lung CD11c⁺ cells. Together, these data suggest that an existing pulmonary mycobacterial infection alters the phenotype of lung dendritic cells so that they can activate antigen-specific naive CD4⁺ T cells in the lungs in response to airway antigen challenge.

The lungs constantly come into contact with airborne particulates and pathogens. The interaction between inhaled particulate antigens, alveolar macrophages, and pulmonary dendritic cells (DCs) sets the stage for antigen-specific pulmonary immune responses (3). In the absence of microbial stimuli, immune responses in the lungs to soluble antigens are characterized by either tolerance or Th2-like responses as seen in murine models of asthma (29, 40). In contrast, acute viral infections give rise to vigorous pulmonary T-cell responses characterized by Th1 cytokine secretion (23, 32). These studies have provided valuable insights into the dynamic role of the pulmonary environment to discriminate airborne insults and generate appropriate T-cell responses. However, few studies have looked at the initiation of naive T-cell responses in the lungs during prolonged mycobacterial infection.

Mycobacterium tuberculosis establishes a latent infection in the vast majority of immunocompetent individuals. Upon inhalation the mycobacteria are phagocytosed principally by alveolar macrophages. M. tuberculosis circumvents phagosomal maturation and establishes a niche for intracellular survival (14). In the ensuing adaptive response mycobacteria are contained in granulomas within which M. tuberculosis persists latently (6). The modulation of pulmonary immunity that permits latency to develop is poorly understood. Mycobacterium bovis bacillus Calmette-Guerin (BCG) is used as a vaccine to prevent disseminated tuberculosis in children; BCG has been used as a model organism to study the innate and adaptive immune response to M. tuberculosis (15, 21, 22). After aerosol BCG infection, pulmonary immune responses and bacterial growth peak 4 to 6 weeks later, followed by gradual clearance of BCG from the lungs (11, 12, 21).

It is thought that activation of naive CD4⁺ T cells, in response to airway antigens, occurs primarily in the mediastinal lymph nodes (MLN) draining the lungs (8, 45). During pulmonary influenza infections, virus-specific naive T cells divide in the MLN, and only the most differentiated cells express the appropriate adhesion molecules to migrate to the lungs (32). In the lungs, differentiated, effector T cells colocalize with antigen-carrying pulmonary DCs (4). However, recent evidence suggests that primary activation of naive T cells can occur in the lungs (24, 34). Mice lacking functional CCL19 and CCL21 and mice lacking fucosyltransferases have impaired localization of naive T cells to secondary lymphoid organs. However, these mice are able to initiate naive T-cell responses in the lungs against pulmonary pathogens. Pulmonary infection may play a role in the apparent shift from draining lymph node to the lung in priming of naive CD4⁺ T cells. Few studies have addressed this issue in their detailed analysis of naive CD4⁺ T-cell responses (16, 27, 44, 45).

In this report, we have used an adoptive-transfer technique (19) to artificially increase the precursor frequencies of ovalbumin (OVA)-specific naive T cells in recipient mice that had been previously infected with aerosolized BCG. Using flow cytometry to track OVA-specific (KJ⁺) T cells, we found that the naive KJ⁺ T-cell response to intranasal OVA was localized to the lungs and draining MLN. Both infected and uninfected mice mounted vigorous OVA-specific T-cell responses in the MLN, but only BCG-infected animals had marked activation.

* Corresponding author. Mailing address: Division of Infectious Diseases, Biomedical Research Building, 1031 Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4984. Phone: (216) 368-4844. Fax: (216) 368-2034. E-mail: whb@cwru.edu.
† W.H.B. and C.V.H. share senior authorship.
‡ Published ahead of print on 12 February 2007.
proliferation, and differentiation of KJ+ T cells in the lungs. Infection caused greater distribution of OVA to pulmonary DCs and enhanced presentation of OVA peptide by lung CD11c+ cells that led to local lung-resident KJ+ T-cell activation and proliferation in vivo.

MATERIALS AND METHODS

Mice. Eight- to ten-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DO11.10 T-cell-receptor (TCR) transgenic mice that express TCRs specific for OVA257-264 peptide presented in the context of I-A^d (31) were a gift from Alan Levine (Case Western Reserve University, Cleveland, OH). Mice were housed in specific-pathogen-free conditions. All studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Aerosol BCG infection. BALB/c mice were exposed to aerosol M. bovis BCG in an inhalation exposure system (Glas Col, Terre Haute, IN) as previously described (21). Day 1 colony counts consistently gave 3,200 ± 1,300 CFU per mouse. Bacterial growth in the lungs peaked 4 to 6 wkss afterwards with 190,000 ± 70,000 CFU per mouse. Bacterial growth in the lung-draining MLN was determined to be 1,300 CFU per mouse at 28 days after infection. Infected mice were used as recipients in adoptive-transfer experiments 4 to 6 weeks postinfection. Uninfected mice in all of the experiments were not mock infected.

Endotoxin depletion of OVA. Endotoxin was removed from OVA (Sigma-Aldrich) by using the protocol of Aida and Pabst (1) with minor modifications. OVA was dissolved in PBS, lyophilized and reconstituted with Triton-X-114 solvent to make a 1 mg/ml solution. The solution was chilled on ice for 10 min and then agitated gently at 4°C for 20 min. The solution was then warmed to 37°C for 10 min and spun at 20,000 × g for 20 min. The detergent phase was aspirated off, and the aqueous phase containing OVA was subjected to seven more extractions with Triton-X-114. The endotoxin contamination was <0.1 µg/ml, as determined by a Limulus amoebocyte lysate assay (BioWhittaker).

DO11.10 T-cell isolation. Splenocytes from 9- to 14-week-old DO11.10 mice were isolated, and red blood cells were lysed in hypotonic lysis buffer (10 mM Tris-HCl and 0.83% ammonium chloride). The cells were plated in 100-mm petri dishes and allowed to adhere for 1 h at 37°C. Nonadherent splenocytes were then used to obtain untouched CD4+ T cells by using the CD4+ T-cell negative selection kit (Miltenyi Biotec) according to the manufacturer’s instruction. In most experiments, the resulting CD4+ T cells were subsequently stained with anti-CD262L and anti-CD44 monoclonal antibodies (MABs) and fluorescence-activated cell sorted (FACS) gated by sorting on naive (CD62L+ CD44hi) T cells by using a BD Aria cell sorter. Purified CD4+ T cells were subsequently stained with anti-CD44 monoclonal antibodies (MABs) and fluorescence-activated cell sorted (FACS) gated by sorting on naive (CD62L+ CD44hi) T cells by using a BD Aria cell sorter. Purified CD4+ T cells and flow-sorted naive CD4+ T cells were then used in adoptive-transfer experiments. FACSorted CD4+ T cells were ≥95% CD44+CD62L+, and 75 to 85% of these naive CD4+ T cells were OVA-specific (KJ+).

Adoptive transfer and ELISPOT OVA challenge. Uninfected BALB/c mice and BCG-infected mice were anesthetized intraperitoneally with a nonlethal dose of tribromoethanol (240 mg/kg) and were then injected with 5 × 10^6 DO11.10 T cells that were pulsed with 100 µg/ml OVA257-264 peptide and washed three times in ice-cold PBS before adoptive transfer in normal saline. Mice were allowed to rest for 2 days before being challenged intranasally on day 0 with 500 µg of endotoxin-depleted OVA or BSA as the control antigen. On day 2, mice were challenged intranasally once more with 500 µg of endotoxin-depleted OVA, while control mice were not given BSA again. On day 3, 5 days after DO11.10 CD4+ T cell transfer, the mice were sacrificed and their spleens, lungs, and MLN were harvested. Bronchoalveolar lavage fluid (BALF) was collected.

Spleen size. For experiments involving CFSE, care was taken to minimize exposure to light. Tissues were harvested and processed as previously described (21). Briefly, mice were anesthetized with a lethal dose of tribromoethanol (240 mg/kg). For each animal, the abdominal cavity was incised, the spleen was harvested, and the mouse was exsanguinated. The thymus was cannulated, and the BALF was collected by three aspirations with 1 ml of PBS. Lungs were perfused with 10 ml of PBS and harvested. The draining MLN were then harvested.

Spleens were homogenized and pressed through a 70-µm-pore-size nylon filter. Red blood cells were lysed in red blood cell lysis buffer. Single cells were resuspended in complete medium (Dulbecco modified Eagle medium, 10% fetal bovine serum [FBS], 0.05 mM 2-mercaptoethanol, 2 mM HEPES, 1 mM sodium pyruvate, 100 mM nonessential amino acids, 100 U of penicillin/ml, and 0.1 mg of streptomycin/ml). Lungs were minced and digested with 125 U of type IV collagenase and 30 U of DNase/ml for 90 min at 37°C. Lung aggregates were drawn through a 18-gauge needle three times before being passed through a 40-µm-pore-size nylon filter. The red blood cells were lysed, and the lungs were resuspended in RPMI. Serial dilutions of lung suspension were plated onto 96-well plates to determine the bacterial CFU counts. MLN were processed through a 70-µm-pore-size nylon filter using the plunger of a 1-ml syringe and then resuspended in RPMI.

Cell staining and percentage of OVA-specific T cells that divided. Single-cell suspensions of tissues were counted. Viability of cells was assessed by trypan blue exclusion. A total of 5 × 10^5 to 1 × 10^6 viable lung, MLN, and spleen cells were preincubated in a 1% BSA-PBS solution of FeCblock (BD Pharmingen) for 15 min at 4°C. The cells were then stained with the DO11.10 TCR clonotypic antibody biotinylated KJ-1-26 (Invitrogen catalog number MM7515-3), along with activation and adhesion markers anti-Cd62L, anti-CD44, and anti-CD69 (eBioscience catalog numbers 25-0621, 12-0441, and 25-0691, respectively) and anti-CD25 (BD Pharmingen catalog number 553075), for 30 min at 4°C. Cells were washed once again with 1% BSA, resuspended in streptavidin-Pacific Blue conjugate (Invitrogen), and incubated for an additional 30 min at 4°C. The cells were washed once again with 1% BSA, and the pellets were resuspended in 0.3 ml of 1% paraformaldehyde in PBS. Stained samples were acquired by using a BD LSR II flow cytometer. Flow cytometry results were analyzed with FlowJo (Tree Star, Inc.) software.

The percentage of OVA-specific T cells that divided was calculated by using the method used to determine the responder frequency as previously described (22). In some instances, each daughter cell generation Ni, characterized by dimmer CFSE labeling, was divided by 2Ni to arrive at the number of precursors or responders that gave rise to those daughters in generation N. The number of undivided CFSEhi T cells was used, along with the sum of the responders, to calculate the fraction of KJ+ T cells that divided or responded after OVA challenge.

Intracellular cytokine staining. Lung cells were stimulated for 5 h with phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml and 1 µg/ml of ionomycin (Sigma-Aldrich) in the presence of 10 µg/ml of brefeldin A (Sigma-Aldrich). The cells were collected and surface stained with KJ-1-26 in the presence of mouse FeCblock (BD Pharmingen) in 2% FBS in 1× PBS staining solution at room temperature. Cells were fixed with 4% paraformaldehyde and stained with allophycocyanin anti-IFN-γ or anti-interleukin-4 (IL-4) MAbs (eBioscience) in the presence of saponin for 30 min. Cells were fixed in 1% paraformaldehyde and acquired within 24 h with a BD LSR II flow cytometer.

BrdU incorporation. Recipient mice were challenged with OVA or BSA. After 3 days, mice received i.v. bromodeoxyuridine (BrdU; 2 mg/mouse) (Sigma-Aldrich) 1 h prior to sacrifice (30). Tissues were harvested and single-cell suspensions made. Cells were surface stained with the DO11.10 T-cell-receptor antibody, KJ-1-26, at room temperature in the presence of mouse FeCblock (BD Pharmingen) in 2% FBS in 1× PBS staining solution and then fixed with 4% paraformaldehyde. Cells were permeabilized with saponin for 30 min at room temperature and incubated with 50 U DNase I at 37°C for 1 h. Diced cells were stained with anti-BrdU MAb in saponin solution. Cells were fixed in 1% paraformaldehyde and acquired within 24 h with a BD LSR II flow cytometer.

ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assay for IFN-γ was done as previously described (21). Briefly, sterile ELISPOT plates (Whatman) were precoated with anti-IFN-γ capture antibody (BD Pharmingen catalog no. 555126) overnight at 4°C at a concentration of 5 µg/ml. The plates were blocked with 1% BSA in PBS for 1 h and washed with PBS before the lung, spleen, and MLN cells from OVA-challenged, BCG-infected, and uninfected mice were added at 5 × 10^6 and 1 × 10^6 cells/well. Some wells received exogenous OVA peptide (OVA257-264, 2 µM), and the cells were incubated for 48 h at 37°C. Plates were washed four times with PBS containing 0.05% Tween 20 and incubated for 4 h at room temperature with biotinylated anti-IFN-γ (BD Pharmingen catalog no. 555410) at a concentration of 2 µg/ml. Plates were washed four times, and bound IFN-γ was detected by using streptavidin-alkaline phosphatase according to the manufacturer’s instructions (R&D Elispot Blue Color Module). Plates were dried at room temperature, and the spots were counted and analyzed by using an immunospot reader and software (CTL Analyzers, LLC, Cleveland, OH). ELISPOT assay for IL-4 was done with a mouse IL-4 ELISPOT kit according to the manufacturer’s instruction (eBioscience). The same cell numbers were plated as described above.

Fluos-OVA preparation and intranasal challenge. Fluos-OVA was prepared by using a fluorescein labeling kit (Roche). Briefly, OVA (Sigma-Aldrich) was dissolved in PBS to make a 10-mg/ml solution. Fluos was dissolved in dimethyl
sulfoxide to make a 2-mg/ml solution. A 95-μl portion of Fluos was added to 2 ml of the OVA solution (10 mg/ml), followed by incubation at room temperature for 2 h with gentle mixing in the dark. Unbound Fluos was separated by using PD-10 columns (GE Healthcare). Then, 450 μg of Fluos-OVA was introduced intranasally into BCG-infected and uninfected mice. After 18 to 24 h the mice were sacrificed, and their lungs and MLN were harvested. Single-cell suspensions were stained with anti-CD11c and anti-CD11b (BD Pharmingen) and either anti-I-A^d, anti-CD80, or anti-CD86 (BD Pharmingen catalog numbers 555346, 553769, and 553691, respectively). The cells were fixed in 1% paraformaldehyde and acquired by using a BD LSR II flow cytometer.

**RESULTS**

**BCG infection causes enrichment and accumulation of OVA-specific T cells in lungs and draining lymph nodes after airway OVA challenge.** CD4^+ T cells from DO11.10 OVA-specific TCR transgenic mice were adoptively transferred into uninfected BALB/c mice and mice that had been infected 4 to 6 weeks earlier.Recipient mice were challenged intranasally on days 0 and 2 with either soluble endotoxin-depleted OVA or BSA as a control antigen. After 3 days of OVA challenge, OVA-specific T cells were identified with the clonotypic MAb KJ 1-26 in the MLN, lung, and spleen. BCG-infected mice had increased recruitment, enhanced T-cell activation and proliferation, or both. To determine the contribution of T-cell activation, we adoptively transferred FACSorted naive (CD62L^hi CD44^low^) CD4^+ T cells from DO11.10 mice into BCG-infected and uninfected BALB/c mice and challenged them intranasally with OVA. The expression of markers associated with T-lymphocyte activation (CD69 and CD25) on OVA-specific T cells in the lungs and draining MLN was analyzed after OVA challenge (Fig. 2). The baseline levels of CD25 and CD69 on naive KJ^+^ T cells present was determined in the MLN, lungs, and spleens of the four different groups. (B) Total KJ^+^ T-cell numbers were calculated by multiplying viable cell counts, determined by trypan blue exclusion, by the percentage of KJ^+^ T cells among viable cells gated from a forward-scatter (FSC) versus side-scatter (SSC) plot during fluorescence-activated cell sorting analysis. This experiment was repeated twice with similar findings. Three mice were included in each group. *, P = 0.02; **, P = 0.01; #, P = 0.004; ##, P = 0.02.
suggested recent T-cell activation. Thus, airway OVA challenge causes naïve KJ+ T-cell activation in the MLN of both infected and uninfected mice but, during pulmonary BCG infection, activation of naïve KJ+ T cells also occurs in the lungs.

**BCG infection increases responder frequency of antigen-specific T cells after airway antigen challenge.** To determine whether differences in activation markers measured in infected and uninfected mice also resulted in differences in T-cell proliferation, CFSE-labeled CD4+ OVA-specific T cells were transferred into infected and uninfected mice. At 3 days after OVA challenge, CFSE dilution of KJ+ T cells in lungs, MLN, and spleens was used as a measure of T-cell proliferation in vivo (25). CFSE dye dilution in KJ+ T cells was antigen specific because no KJ+ T-cell division was observed in animals challenged with BSA (Fig. 3A). In draining MLN and lungs, infected and OVA-challenged mice (BCG+OVA) had greater numbers of divided KJ+ T cells in each generation of daughter cells compared to uninfected OVA challenged mice, even though in both groups KJ+ T cells underwent approximately six cell divisions (Fig. 3A). Proliferation intermediates, corresponding to the first few cell divisions, were prominent in the MLN but not in the lungs of both OVA-challenged groups. This suggested that priming of naïve CD4+ T cells occurs primarily in the MLN but that KJ+ T-cell activation can also occur in infected lungs (Fig. 2B). Very little KJ+ T-cell division was apparent in the spleens, demonstrating again that the antigen-specific T-cell response to an airway antigen was primarily localized to the lungs and draining lymph nodes. We calculated the percentage of OVA-specific T cells that divided by using methods to derive responder frequencies (43) (Fig. 3B). BCG+OVA group of mice had higher responder frequencies of KJ+ T cells than OVA-challenged mice. This difference was less apparent in the MLN than the twofold difference observed in the lungs (Fig. 3B). Enhanced CFSE dilution of KJ+ T cells in the lungs of BCG-infected mice could be due to (i) increased recruitment to the lungs of KJ+ T cells that had divided elsewhere or (ii) local KJ+ T-cell proliferation in the lungs.

**BCG infection results in antigen-specific T-cell proliferation in the lungs upon airway antigen challenge.** To further determine the contribution of in situ pulmonary KJ+ T-cell proliferation in the accumulation of proliferating KJ+ T cells in the lungs (Fig. 3), we adoptively transferred naïve (CD44low CD62Lhi) KJ+ T cells into recipient mice and challenged the mice with 300 μg of endotoxin-depleted OVA. After 3 days of OVA challenge, recipient mice received 2 mg of BrdU intravenously 1 h prior to sacrifice (30). The short pulse allowed us to measure T-cell division in situ with minimal chance for migration. After this short BrdU pulse, BrdU+ OVA-specific T cells were found in the lungs of BCG-infected mice challenged intranasally with OVA (Fig. 4B). In uninfected mice challenged with OVA, fewer BrdU+ KJ+ T cells were found in the lungs (P < 0.05). In animals infected with BCG and challenged with BSA, no BrdU+ KJ+ T cells were detected. A small percentage of BrdU+ KJ+ events within the lungs was due to the short BrdU pulse. We did not detect BrdU+ KJ+ events in the lungs earlier than 3 days after OVA challenge even in BCG+OVA mice. BrdU incorporation was measured in draining MLN 2 days after OVA challenge in both BCG+OVA and OVA mice (data not shown). Thus, BCG infection causes in situ proliferation of antigen-specific T cells in the lungs after airway antigen challenge.

**BCG infection increases the frequency of antigen-specific effector T cells that secrete IFN-γ in the lungs of mice challenged with airway antigen.** To determine whether pulmonary BCG infection increased the differentiation of naïve KJ+ T cells to effector T cells, flow-sorted naïve (CD44low CD62Lhi) OVA-specific T cells were transferred into naïve and BCG-infected mice. Recipient mice were challenged with OVA as described in Materials and Methods. We first determined the fraction of divided KJ+ T cells (CFSEM) in the lungs of infected and uninfected OVA-challenged mice that attained an effector phenotype characterized by loss of L-selectin, CD62Llow (Fig. 5A). L-selectin is a lymph node homing molecule present on naïve and central memory T cells but absent on effector T cells (20, 38). Among KJ+ T cells that had divided more than two times (CFSEM), 49% were CD62Llow in the lungs of BCG+OVA mice, whereas only 23% were

![FIG. 2. BCG infection enhances T-cell activation in the lungs. CD4+ CD62Lhi CD44low FACsorted naïve DO11.10 T cells were transferred into BCG-infected and uninfected mice. Mice were challenged with OVA as described in Materials and Methods. Three days later, the mice were sacrificed, and the draining MLN (A) and lungs (B) were stained for TCR, CD25, and CD69. Histograms were gated on KJ+ T cells. The baseline levels of CD25 and CD69 on naive KJ+ T cells before adoptive transfer are shown in panel A. (C) Percentages of CD25- and CD69-expressing KJ+ T cells, above isotype staining, from three mice per group. *, P < 0.01. The data are representative of three separate experiments.](http://iai.asm.org/Downloaded from on April 30, 2019 by guest)
CD62Llow in the lungs of uninfected OVA-challenged mice. In contrast, 45% of CFSElow KJ+ T cells were CD62Llow in the MLN of uninfected mice, whereas 56% of CFSElow KJ+ T cells were CD62Llow in infected mice. The percentage of CD62Llow KJ+ T cells was greater in the MLN and lungs of infected mice than in uninfected mice (Fig. 5B). Since effector T cells preferentially migrate to sites of inflammation (38), the differences between the two groups were magnified in BCG-infected lungs. Thus, infection causes increased differentiation of activated T cells into effector cells, and this is more prominently observed in the lungs than in the draining lymph nodes.

Next, to determine whether there are differences in cytokine production by OVA-specific effector T cells from lungs of BCG/H11001 OVA and OVA mice, we stimulated lung cells from both groups of mice with PMA and ionomycin and measured the intracellular IFN-γ and IL-4 production. BCG infection increased the frequency of IFN-γ-producing KJ+ T cells among OVA-specific T cells present in the lungs of OVA-challenged mice (Fig. 6A). We did not detect any IL-4-producing KJ+ T cells in either group of mice by intracellular cytokine staining (data not shown). However, stimulating lung T cells with exogenous OVA peptide allowed detection of IL-4-producing cells by ELISPOT assay. As shown in Fig. 6B, similar numbers of IL-4 spot-forming units (SFU) were found between infected and uninfected mice, whereas more IFN-γ SFU were observed in infected and OVA-challenged mice. This demonstrates a Th1 effector phenotype of OVA-specific T cells in the lungs of infected mice.

Lung CD11c+ cells harbor airway-derived OVA and present more OVA peptide in BCG-infected mice than in uninfected mice. One possible explanation behind the increased activation, proliferation, and differentiation of antigen-specific T cells in the lungs of infected mice is that BCG-infected mice express more OVA peptide in lung CD11c+ cells than uninfected mice. This is consistent with the finding that BCG-infected mice express more OVA peptide in lung CD11c+ cells than uninfected mice.

**FIG. 3.** Pulmonary infection increases the frequency of antigen-specific T cells responding to an airway antigen. A total of 5 × 10⁶ CFSE-labeled CD4+ DO11.10 T cells were transferred into BCG-infected and uninfected recipient mice. Recipient mice were challenged with 500 μg of endotoxin-depleted OVA on days 0 and 2 or with BSA on day 0. Mice were sacrificed on day 3, and single-cell suspensions from tissues were stained and analyzed by flow cytometry to measure T-cell proliferation. (A) Representative dot plots showing KJ+ staining and CFSE dye dilution of four individual mice from the four groups. (B) The percentage of OVA-specific T cells that divided in each mouse was calculated as described in Materials and Methods. This experiment was repeated twice with similar results (three mice per group). *, P = 0.02; **, P = 0.03.

**FIG. 4.** BCG infection induces OVA-specific T-cell proliferation in the lungs after airway OVA challenge. FACsorted naive (CD62Lhi CD44low) CD4+ T cells from DO11.10 mice were transferred into BCG-infected and uninfected recipient mice. Recipient mice were challenged intranasally with 300 μg of endotoxin-depleted OVA or BSA. At 3 days after challenge, mice were injected intravenously with 2 mg of BrdU. After 1 h of in vivo BrdU pulse, the mice were sacrificed, and single cell suspensions from the lungs were stained with KJ 1-26 and anti-BrdU as described in Materials and Methods. (A) Isotype staining for anti-BrdU and KJ isotype control. (B) Representative dot plots of three mice from the three groups. The numbers represent the percentage of BrdU+ cells among the KJ+ T cells. (C) Means ± the standard deviation of three mice per group. *, P = 0.01. The experiment was repeated twice with similar findings each time.
cells in the lungs of infected mice is activation of the pulmonary innate immune cells responsible for initiating adaptive responses. CD11c+ lung DCs are efficient at presenting airway antigens to both naive and effector T cells (4). To determine whether CD11c+ lung cells had a role in the increased proliferation and differentiation of KJ1-26+ T cells in the lungs of BCG+OVA mice, CD11c+ and CD11c-DCs were given Fluos-labeled OVA. After 18 to 24 h, the lungs were harvested and stained for phenotypic and maturation markers of DCs. Almost all of the Fluos-OVA was sequestered in the CD11c+ lung cell population (Fig. 7A). This is in agreement with other published findings where intranasally administered antigens had been tracked in vivo (7, 42).

Thus, to determine whether infected and uninfected mice differ in distribution of Fluos-OVA within the CD11c+ lung cell population, we gated on Fluos-CD11c+ cells and measured the DC markers CD11b and CD11c (13). In BCG-infected mice, 19% of cells harboring Fluos-OVA were DCs (CD11bhi CD11chi), whereas in uninfected mice 8.2% of Fluos+ cells were DCs (Fig. 7B). There were greater numbers of CD11bhi CD11chi DCs in the lungs of BCG-infected mice (5%) than in uninfected mice (1%), and this may account for more distribution of OVA to the DCs in infected mice (data not shown). Greater numbers of Fluos-OVA-containing DCs in infected mice could result in activation of more OVA-specific naive T cells (26).

The maturation of lung DCs in infected lungs is demonstrated by high levels of major histocompatibility complex class II (MHC-II; I-Ad) expression (Fig. 7C). Expression levels of the costimulatory molecules CD80 and CD86 were marginally elevated on DCs harboring OVA from infected mice versus uninfected mice (data not shown). Upon infection, greater numbers of Fluos-OVA-containing DCs in infected mice could result in activation of more OVA-specific naive T cells (26).

The activation of naive CD4+ T cells during tuberculosis is demonstrated by high levels of major histocompatibility complex class II (MHC-II; I-Ak) expression (Fig. 7C). Expression levels of the costimulatory molecules CD80 and CD86 were marginally elevated on DCs harboring OVA from infected mice versus uninfected mice (data not shown). Upon infection, greater numbers of CD11c+ MHC-IIbhi cells acquired intranasal antigen and accumulated in the lungs (Fig. 7D). To determine whether increased expression of I-Ak resulted in greater presentation of OVA–I-Ak complexes to KJ1-26+ T cells in infected lungs, CD11c+ lung cells were purified from both infected and uninfected groups of mice using CD11c+ magnetic beads. Exogenous OVA peptide presentation by these CD11c+ lung
cells was assessed by using the DOBW T-cell hybridoma that recognizes OVA\textsubscript{323-339}, the same epitope recognized by KJ\textsuperscript{+} T cells. The hybridoma response was monitored by measuring IL-2 in culture supernatants using ELISA. The addition of exogenous OVA peptide confirmed that lung CD11\textsuperscript{c}\textsuperscript{+} cells from infected mice had more functional MHC-II than cells from uninfected mice, as determined by their efficiency in presenting OVA peptide to DOBW T-cell hybridomas (Fig. 7E). Together, these data suggest that ongoing pulmonary mycobacterial infection increases the number and maturation of lung DCs such that they can activate antigen-specific naive CD4\textsuperscript{+} T cells in the lungs themselves.

**DISCUSSION**

We determined here the effect of prolonged, pulmonary mycobacterial infection on the initiation of naive CD4\textsuperscript{+} T-cell responses. The following specific questions were addressed. (i) Does BCG infection change the distribution of naive CD4\textsuperscript{+} T cells in MLN and lung? (ii) Does pulmonary inflammation promote naive CD4\textsuperscript{+} T-cell recruitment and activation in the lung? (iii) Finally, does inflammation affect CD4\textsuperscript{+} effector T-cell development? Exploring these questions in light of recent reports suggesting that infection affects how and where naive T cells are activated in the lungs was of particular interest (24, 30, 34). BCG-infected mice after airway-OVA challenge had greater accumulation, activation, and proliferation of naive OVA-specific CD4\textsuperscript{+} T cells in the draining MLN than did uninfected mice. Prolonged pulmonary inflammation also was associated with naive CD4\textsuperscript{+} T-cell activation in lung tissue. In contrast, in uninfected mice there was little to no naive CD4\textsuperscript{+} T-cell activation and proliferation in the lungs. Pulmonary BCG infection was associated with decreased expression of L-selectin (CD62L) and increased production of IFN-\gamma by activated OVA-specific CD4\textsuperscript{+} T cells in the lungs. Enhanced CD4\textsuperscript{+} T-cell activation in infected lungs was associated with increased maturation of lung DCs and presentation of exogenous OVA peptide by lung CD11\textsuperscript{c}\textsuperscript{+} cells.

Pulmonary BCG infection allowed measurement of the naive CD4\textsuperscript{+} T-cell responses to an unrelated airway antigen in mice in the presence or absence of prolonged pulmonary inflammation. Pulmonary infection and inflammation peak 4 to 6 weeks after aerosolized BCG infection (21). Naive OVA-specific CD4\textsuperscript{+} T cells were adoptively transferred into uninfected and 4- to 6-week-BCG-infected mice, and then the animals were challenged intranasally with endotoxin-depleted OVA. Previous studies have examined the role of mycobacterial infection of in vitro-generated APCs on naive T-cell activation in vivo (2). Our experimental system allowed for the characterization of naive CD4\textsuperscript{+} T-cell activation by endogenous lung APCs during mycobacterial infection. In addition, we could

![FIG. 7. BCG infection causes upregulation of MHC-II on lung CD11c\textsuperscript{+} cells harboring intranasal Fluos-labeled OVA. BCG-infected and uninfected mice were given 450 \mu g of Fluos-labeled OVA intranasally. After 18 to 24 h the mice were sacrificed, and single-cell lung suspensions were prepared and stained with anti-CD11c, anti-CD11b, anti-I-Ad, anti-CD80, and anti-CD86. (A) FSC versus SSC plots were gated on live cells, and dot plots of individual mice from three groups are shown examining CD11c and Fluos. The percentages represent live cells (FSC versus SSC gate) harboring Fluos-OVA (three mice per group). (B) The plots were gated on Fluos\textsuperscript{+} CD11c\textsuperscript{+} cells. The percentages represent the distribution of Fluos-OVA to DCs (CD11\textsuperscript{c}\textsuperscript{hi} CD11b\textsuperscript{hi}) within the CD11c\textsuperscript{+} population (n = 3). * P = 0.01. (C) Histogram plots show the surface expression of MHC-II on the gated populations shown in panel A containing lung CD11c\textsuperscript{+} cells that harbor Fluos-OVA. The geometric mean fluorescence intensities of I-A\textsuperscript{d} staining are 31,000 ± 5,300 and 1,700 ± 450 for the BCG+OVA and OVA groups, respectively (n = 3). P = 0.03. (D) Total numbers of Fluos\textsuperscript{+} CD11c\textsuperscript{+} cells expressing high I-A\textsuperscript{d} mean fluorescence intensity >3,000; n = 3). ** P = 0.04. (E) CD11c\textsuperscript{+} cells were isolated from the lungs of infected and uninfected mice and plated out at different cell densities along with 2 \mu M OVA peptide and 10\textsuperscript{5} DOBW cells. IL-2 in the culture supernatants was assayed by ELISA. Similar data were obtained in two separate experiments.](http://iai.asm.org/Downloaded from by guest)
control the amount of antigen and the precursor frequency of transferred naive CD4+ TCR transgenic T cells in infected and uninfected mice. Mice were challenged with a high concentration of antigen to maximize access to antigen and minimize clonal competition (5). Our experimental system specifically addressed infection-induced pulmonary modulation of naive CD4+ T-cell priming without measuring pathogen-specific T-cell responses. The latter will vary as pathogen burden and pathogen-derived antigens change during chronic infection (18, 23, 32, 35).

Pulmonary BCG infection increased the trafficking of CD4+ T cells of all antigen specificities to the MLN, as seen in other infection models, but only T cells recognizing cognate antigens remained and proliferated within the MLN (39). In vivo proliferation as measured by CFSE demonstrated that infected mice had a higher T-cell responder frequency than did uninfected mice. CD4+ T-cell proliferation could occur elsewhere, and pulmonary inflammation recruited activated T cells to the MLN. However, the proliferation of OVA-specific T cells was evident in the MLN but not in the lungs or spleens at earlier time points (i.e., fewer than 3 days) after OVA challenge (data not shown). Thus, a greater proliferation of OVA-specific T cells in the MLN of infected mice was responsible for the enhanced accumulation of KJ+ T cells in the MLN.

Our results also suggest that the increased accumulation of KJ+ T cells in the lungs of BCG-infected mice was due to naive CD4+ T-cell activation and proliferation in the lungs. First, BCG-infected mice had CD69+ KJ+ T cells in the lungs, CD69 expression by T cells has a role in inhibiting their egress from lymphoid organs (36). The presence of CD69+ KJ+ T cells in the lungs suggests that these cells may have been activated without migration through the MLN. There were no CD69+ KJ+ T cells in the lungs of uninfected mice even though both infected and uninfected groups had comparable percentages of CD69+ KJ+ T cells in the MLN. Second, short pulses of BrdU in vivo demonstrated that KJ+ T cells proliferated in the lungs of infected mice. Evidence for KJ+ T-cell activation and proliferation in the lungs does not rule out that OVA-specific naive T cells could have been activated elsewhere, migrated to the lungs, and then re-encountered OVA there. However, other researchers have shown that influenza virus infection leads to formation of lymphoid aggregates, bronchus-associated lymphoid tissue, within the lungs of infected mice that facilitate naive T-cell activation and proliferation in the absence of peripheral lymphoid organs (30). In addition, lungs from M. tuberculosis-infected mice express CCL21 and CCL19, naive T-cell chemoattractants, and contain organized neolymphoid structures (34). Secondary lymphoid structures induced by chronic infection could serve as sites of naive CD4+ T-cell priming in the lung.

BCG infection also resulted in increased numbers of effector CD4+ T cells in the MLN and lungs. This difference was more pronounced in the lungs. Increased migration and effector CD4+ T-cell differentiation could explain this finding (7). BCG infection may enhance T-cell activation and effector cell development by causing a greater influx of naive T cells into lymphoid tissues in the mediastinum and lung, as has been demonstrated during herpes simplex virus type 2 infection (37). This increases the likelihood that during BCG infection greater numbers of naive T cells are exposed to cognate antigen presented by DCs in lymphoid tissues. Increased exposure to cognate antigen results in increased naive T-cell activation and division as observed in our experiments (28). In addition, Catron et al., have shown that the presence of greater numbers of naive T cells in lymph nodes at the time of antigen challenge facilitates the generation of effector T cells (5).

The two different pulmonary environments responsible for generating T-cell responses in infected and uninfected mice gave rise to divergent maturation states of lung DCs. BCG can cause DC maturation in vitro by interacting with Toll-like receptors 2 and 4 present on these cells (17, 41). Our studies indicate that pulmonary BCG infection results in the maturation of lung CD11c+ cells harboring airway OVA. Lung CD11c+ cells from infected mice were more capable of presenting exogenous OVA peptide ex vivo than lung CD11c+ cells from uninfected mice. Concomitant with DC maturation, the activation of Toll-like receptors on DCs enhances migration from peripheral sites to draining lymph nodes by upregulating CCR7 (17). The distribution of airway OVA to greater numbers of DCs expressing high levels of MHC-II suggests that mature DCs from infected lungs could migrate to MLN and initiate robust naive OVA-specific T-cell responses. In addition, TLR engagement on DCs induces the production of cytokines such as IL-12 that promote the differentiation of responding T cells to Th1 cells, with the balance between Th1 and Th2 being determined by the strength of the TLR stimulation (9). BCG infection enhanced the differentiation of OVA-specific naive CD4+ T cells in the lungs into Th1 effectors. This is in agreement with previous studies that examined the role of BCG infection in promoting allergen-specific Th1 responses (10, 33).

Therefore, we propose two complementary mechanisms for the initiation of naive CD4+ T-cell responses in the lungs during mycobacterial infection. Pulmonary infection causes lung DC maturation and increases DC trafficking to MLN to initiate robust naive CD4+ T-cell responses in the lymph node. Alternatively, BCG infection causes lung DC maturation and in situ activation of the naive CD4+ T cells in the lungs. Additional studies will determine where naive CD4+ T-cell activation occurs within different lung compartments during chronic inflammation: the alveolar space, the bronchus-associated lymphoid tissue, or the lung parenchyma.

ACKNOWLEDGMENTS

We thank Melanie Campbell for mouse husbandry, Nicole Pecora advised us on the choice of fluorochromes and general flow cytometry techniques. Robert Mahon helped with cytokine assays. David Canaday lent his expertise in depleting endotoxin from ovalbumin and labeling ovalbumin with Fluos. Roxana Rojas provided guidance and suggestions for assays examining DCs. This study was supported by National Institutes of Health grants AI27243 and HL55967 to W.H.B., grants AI34343 and AI35726 to C.V.H., and grant T32 GM07250 to M.M.A.

REFERENCES

1. Aida, Y., and M. J. Pabst. 1990. Removal of endotoxin from protein solutions by phase separation using Triton X-114. J. Immunol. Methods 132:191–195.
2. Bhatt, K., S. P. Hickman, and P. Salgame. 2004. Cutting edge: a new approach to modeling early lung immunity in murine tuberculosis. J. Immunol. 172:2748–2751.
3. Bingisser, R. M., and P. G. Holt, 2001. Immunomodulating mechanisms in the lower respiratory tract: nitric oxide mediated interactions between alveolar macrophages, epithelial cells, and T cells. Swiss Med. Wkly. 131:171–179.
4. Byersdorfer, C. A., and D. D. Chaplin. 2001. Visualization of early APC/T cell interactions in the mouse lung following intranasal challenge. J. Immunol. 167:6756–6764.

5. Catron, D. M., L. K. Rusch, J. Hataye, A. A. Itano, and M. K. Jenkins. 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J. Exp. Med. 203:1045–1054.

6. Chan, J., and J. Flynn. 2004. The immunological aspects of latency in tuberculosis. Clin. Immunol. 110:2–12.

7. Constant, L. S., J. L. Brogdon, D. A. Piggott, C. A. Herrick, I. Visintin, N. H. Ruddell, and K. Bottomly. 2002. Resident lung antigen-presenting cells have the capacity to promote Th2 T-cell differentiation in situ. J. Clin. Invest. 109:1441–1451.

8. Curtis, J. L. 2005. Cell-mediated adaptive immune defense of the lungs. Proc. Am. Thorac. Soc. 2:412–416.

9. Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2004. Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J. Exp. Med. 196:1645–1651.

10. Erb, K. J., J. W. Holloway, A. Sobeck, H. Moll, and G. Le Gros. 1998. Infection of cetaceans with Mycobacterium bovis-Calmette-Guerin bacillus: findings in CETBL/6 mice. Am. J. Respir. Cell Mol. Biol. 22:333–343.

11. Fulton, S. A., T. D. Martin, R. W. Redline, and W. Henry Boom. 2004. Neutrophil-mediated mycobacteriocidal immunity in the lung during Mycobacterium tuberculosis BCG infection in C57BL/6 mice. Infect. Immun. 72:5322–5327.

12. Gonzalez-Juarros, M., and I. M. Orme. 2001. Characterization of murine lung dendritic cells infected with Mycobacterium tuberculosis. Infect. Immun. 69:1127–1133.

13. Honer zu Bentrup, K., and D. G. Rassell. 2001. Mycobacterial persistence: adaptation to a changing environment. Trends Microbiol. 9:597–605.

14. Humphreys, I. R., G. R. Stewart, D. J. Turner, J. Patel, D. Karamanou, R. J. Constant, S. L., J. L. Brogdon, D. A. Piggott, C. A. Herrick, I. Visintin, N. H. Ruddell, A. Itano, and K. A. Pape. 2002. In vivo activation of antigen-presenting cells in the regional lymph nodes of mice following antigen challenge. J. Immunol. 169:11001–11009.

15. Kipnis, A., S. E. Henrickson, and U. H. Von Andrian. 2003. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. Immunity 19:47–57.

16. Iwasaki, A., and E. A. MsgBox/NA. 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5:987–995.

17. Jelley-Gibbs, D. M., R. Redline, and W. Henry Boom. 2000. Pulmonary immune responses during primary Mycobacterium bovis-Calmette-Guerin bacillus infection in C57BL/6 mice. Am. J. Respir. Cell Mol. Biol. 22:333–343.

18. Fulton, S. A., S. M. Reba, T. D. Martin, and W. H. Boom. 2002. Neutrophil-mediated mycobacteriocidal immunity in the lung during Mycobacterium tuberculosis infection. Infect. Immun. 70:1131–1140.

19. Shi, L., R. North, and M. L. Gannu. 2004. Effect of growth state on transcription levels of genes encoding major secreted antigens of Mycobacterium tuberculosis in the mouse lung. Infect. Immun. 72:2420–2424.

20. Tietz, W., and A. Hamann. 1997. The migratory behavior of murine CD4+CD8+ T cells of memory phenotype. Eur. J. Immunol. 27:2225–2232.

21. Topham, D. J., M. R. Castrucci, F. S. Wingo, G. T. Belz, and P. C. Doherty. 2000. The role of antigen in the localization of naive, acutely activated, and memory CD8+ T cells to the lung during influenza pneumonia. J. Immunol. 165:693–6999.

22. Wells, A. D., H. Gudmundsdottir, and L. A. Turka. 2006. Influence of mucosal adjuvants on antigen transport and processing. Nat. Rev. Immunol. 6:51–60.

23. Vermaelen, K. V., L. R. Tietz, W., and A. Hamann. 1997. The migratory behavior of murine CD4+CD8+ T cells of memory phenotype. Eur. J. Immunol. 27:2225–2232.

24. Xia, W., C. E. Pinto, and R. L. Kradin. 1995. The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. J. Exp. Med. 181:1275–1283.