Resident Th1-Like Effector Memory Cells in Pulmonary Recall Responses to *Mycobacterium tuberculosis*

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We recently described a model of Th1 recall responses based on segmental antigen challenge with purified protein derivative of *Mycobacterium tuberculosis* (PPD). Bronchoscopic instillation of 0.5 tuberculin units of PPD resulted in localized lymphocytic inflammation in PPD-positive subjects only. Recruited lymphocytes were predominantly CD4⁺ and were enriched for cells capable of PPD-specific interferon (IFN)- γ production. In the current study, we investigated the mechanisms by which this localized recall response is mobilized. Bronchoscopic PPD challenge of skin test-positive subjects resulted in the production of CXCR3 ligands IFN- γ -inducible protein (IP)-10 and monokine induced by IFN- γ (Mig), but not of CCR5 ligands macrophage inflammatory protein-1 α and regulated-upon activation, normal T-cell expressed and secreted, whereas skin test-negative subjects produced none of these chemokines. Baseline bronchoalveolar lavage (BAL) cells of skin test-positive subjects produced IP-10 and Mig in response to *in vitro* stimulation as well. Because IP-10 and Mig are IFN- γ -inducible chemokines, these findings suggested that chemokine responses to PPD were facilitated by resident memory cells of the lung. Further studies confirmed that baseline BAL lymphocytes of PPD-positive subjects produce IFN- γ in response to PPD, and that, compared with peripheral blood, BAL cells are preferentially enriched for PPD-specific lymphocytes. This IFN- γ production is predominantly a function of CD4⁺ T cells that display the CD45RO⁺/CCR7⁻ surface phenotype characteristic of effector memory cells.

Keywords: chemokines; recall responses; resident memory cells; tuberculosis

The great majority of individuals infected with *Mycobacterium tuberculosis* do not develop active disease, but instead develop specific cell-mediated immunity that is manifest clinically by the development of positive skin-test responses to purified protein derivative of *M. tuberculosis* (PPD) (1). Skin-test responsiveness is associated with relative protection against subsequent infection with *M. tuberculosis* (2), although the mechanisms by which this protection is mediated remain poorly understood. We previously sought to establish a model of *M. tuberculosis*-specific recall responses in the human lung to provide a means to investigate the local responses of immune individuals upon re-exposure

to the organism. To do so, we adapted the technique of bronchoscopic segmental antigen challenge that had previously been used extensively to characterize the local Th2-mediated immune responses in the lungs of individuals with atopic asthma using challenge with allergens such as ragweed (3–5). We instead used PPD as the challenge antigen, and compared responses of naturally infected skin test-positive subjects with those of healthy PPD-negative control subjects. A baseline bronchoalveolar lavage (BAL) was performed, followed by administration of 0.5 tuberculin units (TU) of PPD (i.e., 1/10th of the standard skin-test dose) into the challenged segment and a control instillation of 10 cc of normal saline into a corresponding segment of the contralateral lung. Repeat BAL of control and challenged segments was performed 48 h later.

In our initial studies, we demonstrated that bronchoscopic challenge with PPD resulted in a localized inflammatory response in challenged lung segments of PPD-positive subjects only. Compared with BAL of both baseline and control lung segments of these subjects, PPD-challenged segments displayed a 2.7-fold increase in the total BAL cells and an increase in the percentage of BAL lymphocytes from 10% to 19%. PPD-challenged segments of skin test-positive subjects also displayed an increased percentage of CD4⁺ T cells, and were enriched for cells capable of antigen-specific production of interferon (IFN)- γ in response to *in vitro* stimulation with PPD. In contrast, PPD challenge of skin test-negative subjects resulted in no changes in either total BAL cells or BAL cell differential (6). Our findings thus showed that bronchoscopic challenge with PPD provides a model system that was effective in inducing antigen-specific Th1 recall responses in the human lung.

In the current study, we sought to extend these initial observations by investigating the mechanisms by which antigen-specific Th1-like cells are recruited to the lung in response to PPD challenge. Because Th1 cells characteristically express chemokine receptors CCR5 and CXCR3 (7), we evaluated production of representative CCR5 ligands (macrophage inflammatory protein [MIP]-1 α and regulated-upon activation, normal T-cell expressed and secreted [RANTES]) and CXCR3 ligands (IFN- γ -inducible protein [IP]-10 and monokine induced by IFN- γ [Mig]). We found that BAL fluid from skin test-positive subjects obtained 48 h after PPD challenge contained large amounts of both IP-10 and Mig, but no detectable MIP-1 α or RANTES. In contrast, none of the chemokines studied were detected in post-challenge BAL fluid of skin test-negative subjects. Because both IP-10 and Mig are IFN- γ -inducible chemokines, these findings suggested that PPD-positive subjects could have resident memory cells capable of early IFN- γ production in response to PPD. We then found that baseline BAL cells of PPD-positive subjects produced both IP-10 and Mig following *in vitro* stimulation with PPD, but baseline BAL cells of PPD-negative subjects did not. We determined that antigen-specific IFN- γ -producing cells are present in baseline BAL of PPD-positive subjects. PPD-specific IFN- γ production by baseline BAL cells of skin test-positive subjects is predominantly a function of CD4⁺ T cells that display the CD45RO⁺/CCR7⁻ surface phenotype characteristic of “effector memory” cells (8).

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MATERIALS AND METHODS

Subjects

Bronchoscopy subjects were healthy nonsmoking volunteers aged 18–49 yr. Of the PPD-positive subjects, none had a history of active tuberculosis or of any symptoms suggestive of active tuberculosis, such as cough, night sweats, or weight loss. The PPD-positive group consisted of six males and two females. None of the subjects had a history of vaccination with *Mycobacterium bovis* strain Bacillus Calmette-Guérin (BCG). PPD-positive subjects who underwent bronchoscopic challenge with PPD had been known to have a positive skin test for an average of 6.25 yr (range 2–12 yr) before undergoing the challenge procedure. All other skin test–positive subjects had been known to be PPD-positive for at least 6 mo before their participation in the study. The skin test–negative group consisted of four males and three females. For both PPD-positive and PPD-negative subjects, exclusion criteria included history of asthma or other chronic lung disease, history of cardiac disease, and history of adverse reactions to topical anesthetic agents.

All protocols involving human subjects were approved by the Institutional Board of Review of Case Western Reserve University and University Hospitals of Cleveland.

PPD

Sterile commercially-prepared PPD (Tubersol; Aventis Pasteur, Toronto, ON, Canada) was used in skin testing and bronchoscopic challenge procedures as previously described (6). To assure sterility, dilutions of PPD were prepared in a laminar flow hood immediately before administration using previously unopened vials of Tubersol and sterile physiologic saline.

PPD for *in vitro* use was obtained from the Statens Serum Institut (Copenhagen, Denmark).

Determination of Tuberculin Skin Testing Status

The self-reported PPD skin test status of all subjects was confirmed by performing standard skin-testing in the laboratory. A quantity of 5 TU (0.1 ml) of Tubersol PPD was injected intradermally using 26-G needles. All skin-test responses were measured by one of the investigators (R.S.) as mm of induration 48 h after PPD administration. For the purposes of this study, skin test responses of 10 mm or more of induration were considered positive. Only subjects who displayed no induration in response to 0.5 TU of PPD were admitted to the study as PPD-negative control subjects. Because of the observation that some PPD skin test–negative subjects display *in vitro* responses to antigens of *M. tuberculosis* (9–11), we performed additional *in vitro* screening to further exclude these individuals by incubating peripheral blood mononuclear cells (PBMC) of all PPD skin test–negative subjects with PPD (10 µg/ml) or medium alone for 96 h period as previously described (12). Supernatants of these cultures were then collected and evaluated for PPD-specific IFN- γ production using commercially available ELISA kits (DIF50; R&D Systems, Minneapolis, MN). Only those individuals whose PBMC did not produce significant amounts of IFN- γ in response to PPD were included as subsequent PPD-negative research bronchoscopy subjects.

Isolation of PBMC

Peripheral blood was obtained by venipuncture and mononuclear cells isolated by density sedimentation using Ficoll-Hypaque (Ficoll-Paque PLUS; Amersham Bioscience AB, Uppsala, Sweden). Cells were washed three times in RPMI 1640 (BioWhittaker, Walkersville, MD) and counted using a hemocytometer.

Bronchoscopy Procedures

All bronchoscopies were performed in the Case Western Reserve University General Clinical Research Center (GCRC) at University Hospitals of Cleveland. Pre-procedure lung and cardiac examinations were normal for all subjects. Before each procedure, subjects received aerosolized lidocaine and gargled with 2% lidocaine solution for 60 s. Topical anesthesia of the nasal passage was performed using viscous lidocaine. Further anesthesia was provided by topical application of 1% lidocaine to the airways via the bronchoscope. All subjects were observed in the GCRC for at least 30 min after each bronchoscopy procedure. Subjects

were provided the hospital pager number of one of the investigators and were advised to call if any symptoms of concern arose.

Bronchoscopy with BAL. For procedures involving bronchoalveolar lavage only, the bronchoscope was wedged into a subsegment of the right middle lobe (RML). Lavage was performed by instilling eight 30-cc aliquots of pre-warmed normal saline (NS). Following each instillation, lavage fluid was withdrawn under gentle suction.

Bronchoscopic Challenge with PPD. The procedure of bronchoscopic segmental antigen challenge with PPD has been described in detail previously (6). In brief, the protocol involved two bronchoscopy procedures. In the initial bronchoscopy procedure, BAL of a RML subsegment was performed to determine baseline BAL cell counts. A control instillation of 10 cc of NS was placed into a different subsegment of the RML. The challenge dose of 0.5 TU of PPD was then instilled, in a volume of 10 cc NS, into a subsegment of the lingula. Repeat bronchoscopy was performed 48 h after the challenge procedure. Prior to the procedure, subjects were questioned regarding interval symptoms of cough, sputum production dyspnea, or chest pain, and examination of the heart and lungs was repeated. BAL of the saline control subsegment of the RML was performed first using eight aliquots of 30 cc NS. BAL of the PPD-challenged subsegment of the lingula was then performed, also using eight 30-cc aliquots of NS.

Processing of BAL Fluid

BAL fluid from all procedures was placed on ice for transport to the laboratory. Fluid was aliquoted into 50 cc polypropylene tubes, and the total volume of BAL fluid recovered from each subsegment was recorded. Samples were then immediately centrifuged at $480 \times g$ for 10 min. BAL cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker) with 5% fresh autologous serum and 1% penicillin G (P-3032; Sigma, St. Louis, MO) and counted using a hemocytometer.

Cytospin preparations were made using $\sim 25,000$ – $50,000$ cells from each BAL sample. Resuspended BAL cells were placed in a slide centrifugation apparatus (Cytofunnel, #5991040; Shandon, Pittsburgh, PA) and centrifuged at $800 \times g$ for 8 min in a Shandon Cytospin 3 centrifuge. Slides were then stained with a rapid Wright-Giemsa stain method (LeukoStat, #C430; Fisher Diagnostics, Pittsburgh, PA). Cell differentials were determined by counting 300 cells from each sample under light microscopy.

Assessment of Lymphocyte Subjects

Lymphocyte subsets were analyzed by labeling of samples with fluorescent antibodies (all obtained from Becton-Dickinson, Walkersville, MD). Antibody pairs were selected to allow for identification of CD4+ T cells (CD3+/CD4+, using anti-CD3-PE, #555340 anti-CD4-FITC, #340133), CD8+ T cells (CD3+/CD8+, using anti-CD3-PE as above, and anti-CD8-FITC, #347313), $\gamma\delta$ T cells (CD3+/TCR $\gamma\delta$ +, using anti-CD3-PE and anti-TCR $\gamma\delta$ -FITC, #347903), and natural killer (NK) cells (CD3–/CD56+, using anti-CD3 FITC, #340542, and anti-CD56-PE, #347747). Samples were analyzed by flow cytometry using CellQuest software (Becton-Dickinson). The lymphocyte gate was established by back-gating on CD3+ T cells (from CD3-PE versus forward-scatter plots). Total numbers of lymphocyte populations present in each BAL sample were calculated by multiplying the percentage of specific populations present in the lymphocyte gate by the total number of lymphocytes (as determined by microscopy, above).

Assessment of Chemokine Production

In vivo chemokine responses to bronchoscopic challenge with PPD. *In vivo* chemokine production was assessed using BAL fluid obtained both at baseline and 48 h after bronchoscopic challenge with PPD. Chemokine levels were assessed in nonconcentrated BAL fluid from pre-procedure BAL and from both control and PPD-challenged lung segments using commercially available ELISA kits for IP10 (DIP100; R&D Systems), Mig (DCX900; R&D systems), RANTES (DRN00; R&D Systems) and MIP-1 α (DMA00; R&D Systems). All plates were read at $\lambda = 450$ on an automated plate-reader (Tunable Microplate Reader; Molecular Devices, Sunnyvale, CA) and analyzed using Softmax Pro 3.1.2 software (Molecular Devices).

In vitro production of chemokines by baseline BAL cells. Unsorted BAL cells were resuspended in IMDM with 2% fetal calf serum and

1% penicillin G at concentration 400 K/ml, and aliquoted into 24-well plates (1 ml/well). Cultures were incubated at 37°C for 48 h with PPD (10 µg/ml) or medium alone. Culture supernatants were then aspirated and chemokine concentrations measured using commercially available ELISA kits as noted above.

ELISPOT for IFN- γ

The ability of BAL cells and PBMC to respond to PPD *in vitro* was assessed by ELISPOT for IFN- γ as previously described (6). Briefly, 96-well Unifilter plates (# 7770-0006; Whatman, Clifton, NJ) were coated with the capture antibody anti-human IFN- γ (M-700AE; Endogen, Woburn, MA) and incubated overnight at 4°C. Nonspecific binding was blocked by incubating with 10% fetal calf serum and 1% L-glutamine in RPMI (BioWhittaker) at room temperature for at least 1 h. After rinsing of the blocking solution, PBMC and BAL cells were added to each plate in several concentrations and incubated overnight at 37°C both with medium alone and in the presence of 5 µg/ml of PPD. Either tetanus toxoid (1 µg/ml) or staphylococcal enterotoxin B (1 µg/ml) were used as an additional positive control stimulus. The next day, plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (BP338500; Fisher Scientific, Pittsburgh, PA). Biotin-conjugated anti-IFN- γ (M-701B; Endogen) was then added to each well for an additional overnight incubation at 4°C. After washing, plates were incubated for 2 h at room temperature with peroxidase-conjugated streptavidin (#016-030-084; Jackson ImmunoResearch Laboratories, West Grove, PA). Freshly prepared AEC visualization solution (800 µl of 1% 3-amino-9-ethyl carbazole [A-5754; Sigma], in *N,N*-dimethyl formamide [D-8654; Sigma], added to 24 ml of 0.1 M acetate buffer, pH 5.0) was filtered, and, following addition of 12 µl hydrogen peroxide (Fisher), added to the plate. When spots became visible (~2 min after the addition of the visualization solution), a final rinsing was performed using distilled water. Plates were scanned with CTL ImmunoSpot Plate Scanning Services (Cellular Technology, Ltd., Cleveland, OH), and images analyzed using CTL ImmunoSpot software (Cellular Technology, Ltd.).

Intracellular Staining for IFN- γ and Assessment of BAL Cells Surface Markers

BAL cells were resuspended in IMDM with 5% fresh autologous serum and 1% penicillin G at concentration 400–500 K/ml and aliquoted into 14-ml polypropylene tubes (#352059; Becton-Dickinson). Cells were then incubated with medium alone or PPD (10 µg/ml). Either SEB, 1 µg/ml (S-4881; Sigma), or tetanus toxoid, 4 µg/ml (Wyeth-Ayerst Vaccine, Madison, NJ), was added to an additional tube as a positive control for IFN- γ production. All incubations were performed in the presence of BD Biosciences Reagent Mix (FastImmune CD28/CD49 d #347690; BD Biosciences, San Diego, CA). After an initial 2-h incubation at 37°C, 20 µg/ml Brefeldin A (#347688; BD Biosciences) was added to each tube. All samples were then further incubated 37°C overnight at for a total of 18 h of stimulation.

The next morning, 100 µl 20 mM EDTA was added to each 1-ml sample. After brief vortexing, samples were then incubated for 15 min at room temperature. Cells were then treated with FACS lysis buffer (#347691; BD Biosciences) and incubated for another 10 min at room temperature, then washed in 1% bovine serum albumin/0.1% sodium azide buffer. After centrifugation at 500 $\times$ g for 10 min, cell pellets were resuspended in the wash buffer and aliquoted into microfuge tubes for surface staining. Lymphocyte subsets were identified with the following pairs of surface antibodies (all obtained from BD Biosciences). CD4+ T cells: anti-CD3-APC (#17-0038-71) and anti-CD4-PerCP (#347324); CD8+ T cells: anti-CD3-APC and anti-CD8-PE (#555635); $\gamma\delta$ T cells: anti-CD3-APC and anti- $\gamma\delta$ TCR-PE (#12-9959-73); and NK cells: anti-CD3-APC and anti-CD56-PE (#347747). For CD4+ T cells, memory phenotype was also assessed using additional antibodies anti-CD45RO-APC (#559865) and anti-CCR7-PE (#552176). For all studies, samples of cells were also incubated with appropriate IgG control antibodies of each conjugate to establish proper gating for each antibody. Samples were vortexed, incubated in the dark for 10 min at room temperature, and washed by addition of 1 cc of 1% bovine serum albumin/0.1% sodium azide buffer, followed by centrifugation at 500 $\times$ g for 5 min.

For intracellular staining for IFN- γ , cells were incubated for 10 min

with 0.5 cc of FACS permeability solution (#347692; BD Biosciences) at room temperature and then centrifuged at 500 $\times$ g for 5 min. After removal of supernatants, samples were incubated with anti-IFN- γ -FITC (#340449; BD Biosciences) or, for some tubes, control antibody anti-murine IgG2a-FITC (#11-4729-71; eBioscience, San Diego, CA) in the dark for 30 min at room temperature. Samples were then washed by centrifuge at 500 $\times$ g for 5 min. Supernatants were removed and labeled cells resuspended in 500 µl of 1% paraformaldehyde. Samples were analyzed on a 3-laser flow cytometer (BD LSR; BD Biosciences) using CellQuest software (BD Biosciences).

Statistical Analysis

Comparisons involving multiple studies of the same subjects were performed using paired *t* tests. Comparisons of results of PPD-positive and PPD-negative subjects used unpaired *t* tests. All statistical analysis was performed using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA).

RESULTS

Bronchoscopic Challenge with PPD Stimulates *In Vivo* Production of CXCR3 Chemokine Ligands in Immune Subjects Only

Our previous studies indicated that bronchoscopic challenge with PPD was an effective way to induce localized Th1-like recall responses in the lungs of healthy skin test-positive individuals. To investigate the mechanisms by which these responses are initiated, we first evaluated chemokine responses following PPD challenge. Th1 cells characteristically express chemokine receptors CCR5 and CXCR3. We therefore measured levels of representative chemokine ligands of these receptors in baseline BAL fluid, and from BAL fluid obtained from control and PPD-challenged segments 48 h after bronchoscopic challenge. Findings for four PPD-positive subjects are depicted in Figure 1A. As illustrated, large amounts of CXCR3 ligands IP-10 and Mig were detected in nonconcentrated BAL fluid from PPD-challenged lung segments obtained at 48 h. IP-10 levels in post-challenge BAL fluid were significantly higher than those of baseline BAL ($P = 0.021$) and of BAL obtained from control segments at 48 h ($P = 0.024$). Likewise, Mig levels in post-challenge BAL were significantly higher than those of baseline BAL ($P = 0.003$) and of control segments ($P = 0.040$). In contrast, representative ligands of CCR5 were not present in BAL fluid after PPD challenge. As illustrated, levels of MIP-1 α and RANTES in challenged segments were not significantly different from those of baseline BAL ($P = 0.148$, $P = 0.196$, respectively).

Because production of CXCR3 ligands is dependent upon IFN- γ , we hypothesized that skin test-negative subjects, lacking mycobacteria-specific lymphocytes, may have been unable to produce these chemokines in response to PPD challenge. As illustrated in Figure 1B, this was in fact the case. In PPD-negative subjects, levels of IP-10, Mig, and MIP-1 α all were not significantly different from those of pre-challenge BAL ($P = 0.205$, $P = 0.074$, $P = 0.197$, respectively). RANTES was not detected in the BAL fluid of these subjects under any of the study conditions.

CXCR3 Chemokine Ligands Are Produced by Baseline BAL Cells after *In Vitro* Stimulation with PPD

Because BAL chemokine production after PPD challenge was assessed only at 48 h (by which time recruitment of lymphocytes to the lung had already occurred), we sought to clarify whether cells present in the lung at baseline had the capacity to produce CXCR3 ligand chemokines. Baseline BAL cells from both skin test-positive and skin test-negative subjects were incubated for 48 h with medium alone and with PPD (10 µg/ml). As shown in Figure 2, baseline BAL cells from PPD-positive subjects produced significant amounts of both Mig and IP-10 in response to PPD, whereas those of PPD-negative subjects did not. In response

Figure 1. Segmental antigen challenge with PPD stimulated local production of IP-10 and Mig in skin test-positive subjects only. Nonconcentrated BAL fluid of PPD-positive subjects contains large amounts of CXCR3 chemokine ligands IP-10 and Mig, but not CCR5 ligands MIP1 α and RANTES (A). In contrast, BAL fluid of PPD-negative subjects did not contain measurable amounts of any of the chemokines measured (B). Chemokines were measured in BAL fluid obtained before PPD challenge (white bars), and 48 h after instillation of saline into control segments (striped bars) and of 0.5 TU of PPD into challenged segments (black bars).

to *in vitro* stimulation with PPD, skin test-positive subjects produced 1,960 pg/ml of Mig ($\pm$ 1,425) compared with 190 pg/ml ($\pm$ 159) after incubation with medium alone. In contrast, Mig levels in cultures of baseline BAL cells from skin test-negative subjects cultured with PPD were 68.8 pg/ml ($\pm$ 41) and 4.55 pg/ml ($\pm$ 7.1) with medium alone (Figure 2A). Likewise, BAL cells of skin test-positive subjects produced 1,429 pg/ml ($\pm$ 306) of IP-10 in response to PPD, compared with 171 pg/ml ($\pm$ 133) from culture with medium alone, whereas culture of BAL cells from skin test-negative subjects with PPD or medium alone induced only 136.6 pg/ml ($\pm$ 172.5) and 23.2 pg/ml ($\pm$ 31) of IP-10, respectively. *In vitro* production of IFN- γ -dependent CXCR3 chemokines by baseline BAL cells of PPD-positive subjects was therefore significantly greater than that of skin test-negative subjects with regard to both Mig (P = 0.042 by *t* test) and IP-10 (P = 0.002).

Figure 2. IP-10 and Mig are produced by baseline BAL cells of skin test-positive subjects in response to *in vitro* stimulation with PPD. White bars indicate chemokine levels in supernatants of unstimulated cell cultures, whereas black bars show chemokine levels in cultures stimulated with PPD. After 48 h of *in vitro* stimulation with PPD, resident alveolar cells obtainable by BAL of skin test-positive subjects have the ability to produce IFN- γ -dependent chemokines Mig (A) and IP-10 (B), whereas those of PPD-negative subjects do not. Figures show mean and SD values (n = 3 for each subject group).

Baseline BAL Cells of PPD Skin Test-Positive Subjects Display PPD-Specific Production of IFN- γ

To further assess the ability of resident BAL cells to initiate antigen-specific production of IFN- γ -dependent chemokines, we evaluated the ability of baseline BAL cells isolated from PPD-positive and PPD-negative subjects to produce IFN- γ in response to PPD using ELISPOT. BAL cells were incubated overnight with medium alone and with PPD (5 μ g/ml). Results were expressed as IFN- γ -producing cells per 10,000 total BAL cells. As shown in Figure 3, a mean of 31.0 cells per 10,000 BAL cells from PPD-positive subjects produced IFN- γ in response to *in vitro* stimulation with PPD, whereas PPD-specific production of IFN- γ was observed in only 1.01 cells per 10,000 BAL cells obtained from PPD-negative subjects (P = 0.008). When correlated with the cell differential of each subject's BAL cells, 2.61% of BAL lymphocytes of PPD-positive subjects produced IFN- γ

Figure 3. Baseline BAL cells of PPD-positive subjects display antigen-specific production of IFN- γ in response to PPD. ELISPOT for IFN- γ was performed using baseline BAL cells from both PPD-positive and PPD-negative subjects as described in the text. *White bars* indicate responses of unstimulated cells and *black bars* represent responses of cells incubated with PPD. PPD-positive subjects displayed significantly more IFN- γ -positive cells in response to stimulation with PPD than did skin test-negative subjects. For the later group, IFN- γ production in response to PPD was not significantly greater than that observed in cells cultured in medium alone. Results are expressed as number of IFN- γ -producing cells per 10 K total BAL cells ($n = 4$ for each group).

in response to PPD, compared with 0.19% of BAL lymphocytes of skin test-negative subjects ($P = 0.002$).

Comparison with ELISPOT data from PBMC of the same PPD-positive subjects indicated that PPD-specific IFN- γ -producing cells were selectively localized to the lung. For these subjects, only $3.99 (\pm 2.90)$ of each 10,000 PBMC produced IFN- γ in response to PPD, compared with background production of IFN- γ of $0.25 (\pm 0.31)$ cells per 10,000 unstimulated PBMC. The difference between the frequency of PPD-specific IFN- γ production by blood and lung cells is even more striking when expressed as percentage of lymphocytes that display responses to PPD. Based on typical findings that 80–90% of PBMC are lymphocytes, the ELISPOT data suggests that only 0.44–0.62% of blood lymphocytes of these skin test-positive subjects display PPD-specific production of IFN- γ . In comparison with the observation that 2.61% of BAL lymphocytes produce IFN- γ in response to PPD, these findings indicate an ~ 40 - to 60-fold enrichment for PPD-specific lymphocytes in BAL as compared with peripheral blood.

PPD-Specific Production of IFN- γ by Baseline BAL Cells of Immune Subjects Is Predominantly a Function of CD4+ T Cells

To determine which lymphocyte subsets were sources of PPD-specific IFN- γ production in baseline BAL, we performed intracellular staining for IFN- γ of BAL cells incubated overnight with PPD. Surface staining for lymphocyte subsets was performed as well using CD3/CD4 staining for CD4+ T cells, CD3/CD8 staining for CD8+ T cells, CD3/ $\gamma\delta$ TCR for $\gamma\delta$ T cells, and the combination of lack of staining for CD3 and positive staining for CD56 for NK cells.

For four PPD-positive subjects studied, 58.6% (± 9.8) of BAL lymphocytes were CD4+ T cells, 25.5 (± 9.6) CD8+ T cells, 4.2% (± 1.4) $\gamma\delta$ T cells, and 11.5% (± 4.7) NK cells. Figure 4A illustrates the mean number of BAL lymphocytes of each subset, as well as the portion of cells in each subset that displayed positive intracellular staining for IFN- γ after stimulation with PPD. As illustrated, 10.7% (± 3.3) of CD4+ T cells displayed IFN- γ production in response to PPD, as did 0.8% (± 0.0) of CD8+ T cells, 17.9% (± 8.1) of $\gamma\delta$ T cells, and 4.7% of NK cells.

These data were also re-expressed in terms of the contribution of the various baseline BAL lymphocyte populations to PPD-

Figure 4. CD4+ T cells are the predominant source of antigen-specific IFN- γ production in response to PPD. Production of IFN- γ by baseline BAL cells was assessed using intracellular staining for IFN- γ in combination with surface markers for specific lymphocyte subsets as described. (A) Mean data of total BAL lymphocytes expressing surface markers of the various subsets for four PPD-positive subjects studied. In this stacked bar format, *black portions* of each bar indicate lymphocytes that were negative for IFN- γ production, whereas *white portions* of each bar indicate lymphocytes that displayed positive intracellular staining for IFN- γ after PPD stimulation. (B) Re-expresses these data in terms of the percentage of all IFN- γ -producing lymphocytes that were accounted for by each lymphocyte subset. CD4+ T cells accounted for over 80% of the BAL lymphocytes producing IFN- γ in response to stimulation with PPD, as illustrated.

specific production of IFN- γ , as displayed in Figure 4B. As illustrated, of BAL lymphocytes that displayed positive intracellular staining for IFN- γ after stimulation with PPD, 80.4% were CD4+ T cells. CD8+ T cells represented only 2.8% of PPD-specific IFN- γ -producing cells, whereas 8.8% of IFN- γ -producing cells were $\gamma\delta$ T cells and 8.0% were NK cells.

Baseline CD4+ BAL T Cells that Produce IFN- γ in Response to PPD Display Effector Memory Cell Phenotype

The finding that antigen-specific CD4+ T cells present in BAL at baseline could rapidly produce IFN- γ in response to PPD suggested that these cells were serving in an effector memory role. To confirm this phenotype, we studied expression of lymphocyte surface markers CD45RO and CCR7 on BAL lymphocytes stimulated *in vitro* with PPD. Representative results are displayed in Figure 5. As shown in the example illustrated in Figure 5A, the overwhelming majority of BAL cells expressed CD45RO. For four subjects studied, 92.9% ($\pm 5.0\%$) of BAL lymphocytes expressed CD45RO. Figure 5B demonstrates surface expression of CCR7 on BAL CD4+ T cells that express CD45RO. Of this population, IFN- γ production in response to PPD is nearly exclusively a function of cells that are negative for CCR7. For the same four subjects, a mean of 97.5% ($\pm 1.7\%$) of IFN- γ -producing memory CD4+ T cells were CCR7-negative. The population of baseline CD4+ BAL T cells that produced IFN- γ in response to PPD therefore displayed the CD45RO+/CCR7- surface phenotype typical of effector memory lymphocytes.

DISCUSSION

Recall responses of cell-mediated immunity involve restimulation of antigen-specific memory cells. T cells expressing the memory marker CD45RO have been further categorized as either “central memory” cells or “effector memory” cells. Central memory cells require further stimulation to induce differentiation to produce Th1 or Th2 cytokine profiles. These cells express CCR7 and reside predominantly in lymph nodes. In contrast, peripheral memory is predominantly provided by CCR7-negative effector memory cells. These cells have already developed a profile of cytokine responses and are capable of rapid cytokine production upon restimulation (8).

In this study, we demonstrated that PPD-positive subjects produce IFN- γ -inducible CXCR3 ligands IP-10 and Mig in response to bronchoscopic challenge with PPD, whereas skin test-negative subjects do not. Baseline BAL cells of skin test-positive subjects produce these CXCR3 chemokine ligands in response to *in vitro* stimulation with PPD, but those of skin test-negative individuals do not. Consistent with this observation, baseline BAL cells of

PPD-positive subjects display antigen-specific production of IFN- γ in response to PPD. Approximately 80% of BAL lymphocytes that produce IFN- γ in response to PPD are CD4+ T cells. These Th1-like cells express CD45RO but not CCR7, and therefore express the phenotype of effector memory lymphocytes.

Schwander and coworkers previously reported that BAL findings of PPD-positive subjects did not differ from those of skin test-negative subjects in terms of BAL total cell count, cell differential, or CD4/CD8 ratio. The authors also found that BAL cells from patients with active tuberculosis demonstrated Th1 cytokine responses to *M. tuberculosis* and its antigens (13). The significance of the latter finding in the face of ongoing antigen exposure is unclear, however, other than its demonstration that local IFN- γ production does not itself protect against disease.

Previous studies by the same investigators indicated that PPD-specific IFN- γ -producing T cells were present in BAL of household contacts of patients with tuberculosis (14). In this setting, this presence of such cells could be attributed to recent or ongoing exposure to *M. tuberculosis*. In murine models of infection with various respiratory viral pathogens, persistence of antigen-specific memory cells within the lungs for several months following infection has been documented (15, 16). In humans, remarkably high numbers of resident Th1 cells with specificity for beryllium have been observed in individuals with berylliosis (17). Because of the trapping of inhaled antigen within the lung, these patients have continued local exposure to beryllium. In contrast, the state of latent *M. tuberculosis* infection assumed in healthy PPD-positive subjects is not characterized by the presence of large numbers of viable tubercle bacilli (18). Many of our subjects had documented skin-test positivity for several years before their participation in this study. The presence of antigen-specific Th1-like memory cells in the lungs of these individuals is therefore distinct from these earlier reports. Our findings suggest that previous aerosol exposure to *M. tuberculosis* may result in development of long-lasting and localized Th1-like memory within the lung that persists even without continued high-level exposure to antigen.

Both animal and human studies have indicated an essential role of IFN- γ in protection against mycobacterial infections (19–21). Despite its role as a bactericidal “macrophage activating factor” for other intracellular infections, however, numerous studies have indicated that IFN- γ does not directly mediate killing of intracellular *M. tuberculosis* within human phagocytes (22–24). This paradox implies that IFN- γ , although essential for protection, contributes to the containment of *M. tuberculosis* infection via more indirect means. Our findings suggest that one such role for IFN- γ in human immunity to *M. tuberculosis* may be its sentinel function in localized immunity within the lung,

Figure 5. IFN- γ -producing CD4+ BAL T cells of PPD-positive subjects display memory-effector cell phenotype. BAL cells were incubated overnight with PPD. Intracellular staining for IFN- γ was performed, as was surface staining for various markers as described in MATERIALS AND METHODS. Representative results gated on CD4+ lymphocytes are shown. (A) Assessment of intracellular IFN- γ (x axis) and expression of the memory marker CD45RO (y axis) indicates that 94.7% of CD4+ T cells that produce IFN- γ in response to PPD express CD45RO. (B) Assessment of intracellular IFN- γ (x axis) and expression of CCR7 (y axis) of CD45RO+ CD4 T cells indicates that 99.3% of memory CD4+ T cells capable of PPD-specific IFN- γ production are negative for CCR7.

by which Th1-like memory cells that are preferentially localized to the lung mediate early recruitment of additional CD4⁺ T cells and mononuclear phagocytes to the site of infection. This possibility is consistent with studies examining the beneficial effects of inhaled IFN- γ as an adjunct to treatment of multidrug-resistant *M. tuberculosis* (25). The investigators have determined that treatment with inhaled IFN- γ serves to increase expression of RNA for the chemokine IP-10 by BAL cells, but does not stimulate the pathway for synthesis of the effector molecule nitric oxide (26). The importance of IFN- γ -dependent chemokines in providing for effective cell-mediated immunity within the lung is also supported by murine models of fungal infection. These studies have indicated that the switch within the alveoli from production of IFN- γ -independent to IFN- γ -dependent chemokines is associated with a change in local inflammation from a neutrophil-predominant, nonprotective response to a mononuclear infiltrate that is associated with control of the infection (27).

Our findings indicate that long-lived *M. tuberculosis*-specific Th1 effector memory cells are preferentially localized within the lungs of healthy PPD-positive individuals with a history of respiratory exposure to *M. tuberculosis*, and that these cells function as the major initial source for IFN- γ production within the lung following local re-exposure to protein antigens of *M. tuberculosis*. The ability of baseline BAL cells to produce CXCR3 ligand chemokines IP-10 and Mig *in vitro* demonstrates that production of IFN- γ -dependent chemoattractants can be initiated by these resident cells alone. The much higher chemokine levels observed *in vivo* after bronchoscopic administration of PPD may indicate that further contributions to CXCR3 chemokine production are made by additional cell populations, such as respiratory epithelium (28), or inflammatory cells recruited to the lung during the initial 48 h after challenge. Nevertheless, our findings suggest that resident effector memory Th1 cells may play a critical role in initiating chemokine production and in subsequent recruitment of additional, localized Th1 recall responses to the human lung. The persistence of these memory cells after the original exposure may allow them to play a significant role in providing long-term cell-mediated immunity to inhaled pathogens. Different approaches to vaccination have been shown to alter the degree to which antigen-specific memory cells are localized to the lung (29). Further assessment of these responses may therefore provide a means to evaluate and optimize vaccination strategies aimed at preventing infection with *M. tuberculosis* and other respiratory pathogens controlled by cell-mediated immunity.

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References

- Schlager N, Rom WN. The host immune response to tuberculosis. *Am J Respir Crit Care Med* 1998;157:679–691.
- Stead W. Management of health care workers after inadvertent exposure to tuberculosis: a guide for the use of preventative therapy. *Ann Intern Med* 1995;122:906–912.
- Calhoun W, Jarjour NN, Gleich GJ, Stevens CA, Busse WW. Increased airway inflammation with segmental versus aerosol antigen challenge. *Am Rev Respir Dis* 1993;147:1465–1471.
- Jarjour N, Peters SP, Djukanovic R, Calhoun WJ. Investigative use of bronchoscopy in asthma. *Am J Respir Crit Care Med* 1998;157:692–697.
- Metzger W, Richerson HB, Worden K, Monick M, Hunninghake GW. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs: description of the model and local airway inflammation. *Am Rev Respir Dis* 1987;135:433–440.
- Silver R, Zukowski L, Kotake S, Li Q, Pozuelo F, Krywiak A, Larkin R. Recruitment of antigen-specific Th1-like responses to the human lung following bronchoscopic segmental challenge with purified protein derivative of *Mycobacterium tuberculosis*. *Am J Respir Cell Mol Biol* 2003;29:117–123.
- Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR. The chemokine receptors CXCR3 and CCR5 mark subsets of T-cells associated with certain inflammatory reactions. *J Clin Invest* 1998;101:746–754.
- Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999;401:708–712.
- Ravn P, Boesen H, Pedersen BK, Andersen P. Human T cell responses induced by vaccination with *Mycobacterium bovis* bacillus Calmette-Guerin. *J Immunol* 1997;158:1949–1955.
- Hoft D, Brown R, Roodman S. A double-blind, placebo-controlled study of mycobacterium-specific human immune responses induced by intradermal bacille Calmette-Guerin vaccination. *J Lab Clin Med* 1999;134:244–252.
- Hoft D, Worku S, Kampmann B, Whalen CC, Ellner JJ, Hirsch CS, Brown RB, Larkin R, Ling Q, Yun H, et al. Investigation of the relationship between immune-mediated inhibition of mycobacterial growth and other potential surrogate markers of protective *Mycobacterium tuberculosis* immunity. *J Infect Dis* 2002;186:1448–1457.
- Silver R, Wallis RS, Ellner JJ. Mapping of T cell epitopes of the 30-kD antigen of *Mycobacterium bovis* strain Bacillus Calmette Guerin in purified protein derivative (PPD) positive individuals. *J Immunol* 1995;154:4665–4674.
- Schwander S, Torres M, Sada E, Carranza C, Ramos E, Tary-Lehmann M, Wallis RS, Sierra J, Rich EA. Enhanced responses to *Mycobacterium tuberculosis* antigens by human alveolar lymphocytes during active pulmonary tuberculosis. *J Infect Dis* 1998;178:1434–1445.
- Schwander SK, Torres M, Carranza C, Escobedo D, Tary-Lehmann M, Anderson P, Toossi Z, Ellner JJ, Rich EA, Sada E. Pulmonary mononuclear cell responses to antigens of *Mycobacterium tuberculosis* in healthy household contacts of patients with active tuberculosis and healthy controls from the community. *J Immunol* 2000;165:1479–1485.
- Hogan R, Usherwood EJ, Zhong W, Roberts AD, Dutton RW, Harmsen AG, Woodland DL. Activated antigen-specific CD8⁺ T-cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* 2001;166:1813–1822.
- Hogan R, Zhong W, Usherwood EJ, Cookenham T, Roberts AD, Woodland DL. Protection from respiratory virus infections can be mediated by antigen-specific CD4⁺ T cells that persist in the lungs. *J Exp Med* 2001;193:981–986.
- Foutnot A, Cavanera SJ, Gharavi L, Newman LS, Kotzin BL. Target organ localization of memory CD4⁺ T-cells in patients with chronic beryllium disease. *J Clin Invest* 2002;110:1473–1482.
- Manabe Y, Bishai WR. Latent *Mycobacterium tuberculosis*-persistence, patience, and winning by waiting. *Nat Med* 2000;6:1327–1329.
- Flynn J, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon γ in resistance of *Mycobacterium tuberculosis* infection. *J Exp Med* 1993;178:2249–2254.
- Dorman SE, Holland SM. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. *J Clin Invest* 1998;101:2364–2369.
- Elloumia-Zghal H, Barbouche MR, Chemli J, Bejaoui M, Harbi A, Snoussi N, Abdelhak S, Dellagi K. Clinical and genetic heterogeneity of inherited autosomal recessive susceptibility to disseminated *Mycobacterium bovis* bacille calmette-guerin infection. *J Infect Dis* 2002;185:1468–1475.
- Douvas GS, Crowle AJ. γ -Interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. *Infect Immun* 1985;51:1–8.
- Rook GAW, Steele J, Ainsworth M, Champion BR. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant γ -interferon on human monocytes and murine peritoneal macrophages. *Immunology* 1986;59:333–338.
- Silver RF, Li Q, Boom WH, Ellner JJ. Lymphocyte-dependent inhibition of growth of virulent *Mycobacterium tuberculosis* H37Rv within human monocytes: requirement for CD4⁺ T-cells in purified-protein derivative-positive subjects, but not in purified protein derivative-negative subjects. *J Immunol* 1998;160:2408–2417.

25. Condos R, Rom WN, Schluger NW. Treatment of multidrug-resistant pulmonary tuberculosis with interferon-gamma via aerosol. *Lancet* 1997;349:1513–1515.
26. Raju B, Hoshino Y, Kuwabara K, Belitskaya I, Prabhakar S, Canova A, Gold JA, Condos R, Pine RI, Brown S, *et al.* Aerosolized gamma interferon (IFN- γ) induced expression of the genes encoding the IFN- γ -inducible 10 kilodalton protein but not inducible nitric oxide synthase in the lung during tuberculosis. *Infect Immun* 2004;72:1275–1283.
27. Souto J, Aliberti JC, Campanelli AP, Livonesi MC, Maffei LML, Ferreira BR, Travassos LR, Martinez R, Rossi MA, Silva JS. Chemokine production and leukocyte recruitment to the lungs of *Paracoccidioides brasiliensis*-infected mice is modulated by interferon γ . *Am J Pathol* 2003;163:583–590.
28. Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA, Hamid Q, Luster AD. The T-cell specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. *J Immunol* 1999;162:3549–3558.
29. Palendira U, Bean AGD, Feng CG, Britton WJ. Lymphocyte recruitment and protective efficacy against pulmonary mycobacterial infection are independent of the route of prior *Mycobacterium bovis* BCG immunization. *Infect Immun* 2002;70:1410–1416.