Lymphocyte Recruitment and Protective Efficacy against Pulmonary Mycobacterial Infection Are Independent of the Route of Prior Mycobacterium bovis BCG Immunization

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*Mycobacterium tuberculosis* infects humans through the lung, and immunity to this chronic infection is mediated primarily by CD4+ T lymphocytes. Recently we have demonstrated that the recruitment of lymphocytes to the lung during primary aerosol *M. tuberculosis* infection in mice occurs predominately through the interaction of αβ integrin on CD4+ T cells and vascular cell adhesion molecule-1 on the pulmonary endothelium. To investigate the effect of route of immunization with *Mycobacterium bovis* BCG on the pattern of T-cell recruitment to the lung, we have analyzed the differences in expression of integrins on activated memory CD4+ T cells infiltrating the lung following primary BCG immunization by aerosol, intravenous, and subcutaneous routes and after subsequent aerosol challenge with *M. tuberculosis*. There were marked differences in the patterns of recruitment of activated CD4+ T cells to the lung following primary immunization by the three routes. Expansion of CD44hi CD62Llow CD4+ T cells in the lung occurred following aerosol and intravenous BCG immunizations, and the lymphocyte recruitment was proportional to the pulmonary bacterial load. The majority of infiltrating CD4+ T cells expressed αβ integrin. On subsequent exposure to aerosol BCG rapid expansion of gamma interferon-secreting αβ+ CD4+ T cells occurred to the same extent in all immunized mice, regardless of the route of immunization. Similar expansion of αβ+ CD4+ memory T cells occurred following *M. tuberculosis* challenge. The three routes of BCG immunization resulted in the same level of protection against aerosol *M. tuberculosis* or BCG challenge in both the lungs and spleen. Therefore, recruitment of effector T lymphocytes and protective efficacy against pulmonary mycobacterial infection are independent of the route of prior BCG immunization.

With a third of the world’s population infected with tuberculosis (TB) and large variability in the protective efficacy of the currently approved vaccine BCG, an attenuated strain of *Mycobacterium bovis* BCG (13), there is an urgent need for a new anti-TB vaccine or improvement in delivery of the current vaccine. BCG has been administered by an intradermal route to more than 70% of children worldwide in the last 70 years (2, 14), but it has had little impact on the prevalence of TB (13). Several reasons have been advanced to explain the variable efficacy of BCG against pulmonary TB, including the dose of immunization (23), the strain of BCG used (21, 22), and the confounding effect of prior exposure to environmental mycobacteria (8). An additional factor may relate to the capacity of intradermal BCG immunization to stimulate protective cellular immunity within the lung. A number of studies have provided contradictory evidence on the effect of varying the route of BCG immunization on its protective efficacy against aerosol infection using a variety of animal models. Early reports indicated a greater degree of protection against an aerosol challenge with *Mycobacterium tuberculosis* from BCG vaccination delivered by an aerosol route compared to the subcutaneous (s.c.) route (3, 23). Later work involving adoptive transfer experiments concluded that aerogenic vaccination with BCG offered no immunologic advantage over s.c. vaccination against *M. tuberculosis* (25). None of these studies, however, examined the immunological mechanism of any effect and, in particular, the process of recruitment of effector T lymphocytes to the lung.

Cell-mediated immunity to *M. tuberculosis* is critically dependent on the recruitment of antigen-specific T cells to the lung (7, 10). Both CD4+ and CD8+ T cells play a role in protection against *M. tuberculosis* (15), but the major protective subset of lymphocytes are CD4+ T cells, and thus these shall be the focus of this study. Mice deficient in CD4+ T cells, either due to the lack of CD4 (7) or class II molecules (18), succumb to *M. tuberculosis* and BCG, respectively. Furthermore, humans with CD4+ T-cell deficiency due to human immunodeficiency virus infection have markedly increased susceptibility to *M. tuberculosis* and *M. avium* (17). Adoptive transfer of CD4+ T cells from *M. tuberculosis*-infected mice or BCG-immunized mice to T-cell-deficient mice resulted in protection against *M. tuberculosis* (24) and aerosol BCG infection (11), respectively. CD4+ T cells produce gamma interferon (IFN-γ) which synergizes with tumor necrosis factor to stimulate mycobacterial killing by macrophages (5). As the microenvironment of the site of initial antigen encounter can influence the type of response generated and the acquisition of defined homing molecules, the site of BCG immunization may...
influence the immune response to *M. tuberculosis* in the lung. Immunization by systemic routes stimulates effector memory T cells expressing the integrin α4β7 (26, 30). These cells are recruited to sites of inflammation by binding to vascular cell adhesion molecule-1 (VCAM-1) on activated endothelia (26, 30). By contrast, antigen encounter by mucosal routes leads to the development of memory T cells expressing α4β7, which home to mucosal sites where mucosal addressin cell adhesion molecule (MAdCAM) is expressed on endothelia (28, 29). The lung is often considered to be part of the mucosal system, and immunization by a mucosal route may enhance recruitment of T cells during subsequent exposure to aerosol mycobacteria. Immunization with BCG by different routes could influence the pattern of T-cell recruitment to the lung during subsequent challenge to aerosol infection. Recently, however, the majority of the infiltrating CD4+ T cells in the lungs during *M. tuberculosis* infection were found to express the α4β7, and not the α4β7 integrin (12). Further, there was increased expression of the VCAM-1, but not MAdCAM-1, on pulmonary endothelia during *M. tuberculosis* infection, suggesting that α4β7+ CD4+ T cells were sufficient for protective immunity. Therefore, we hypothesized that activation of α4β7+CD4+ T cells by BCG immunization by different routes would permit the recruitment of these T cells to the lungs on subsequent aerosol challenge with *M. tuberculosis* with resulting protection.

In order to test this hypothesis we have examined the pattern of integrin expression on T cells recruited to the lung during primary BCG immunization by intravenous (i.v.), s.c., and aerosol routes and then following aerosol challenge with BCG or *M. tuberculosis*. While there were marked differences in the number of activated memory T cells expressing α4β7 integrin in the lung after the primary immunizations, there was a similar degree of expansion of α4β7+ T cells in the lung following BCG or *M. tuberculosis* challenge, regardless of the route of prior immunization, with similar levels of protection.

**MATERIALS AND METHODS**

**Mice.** Six- to eight-week-old female C57BL/6 (H-2b) mice were obtained from the Australian Resources Centre (Perth, Western Australia, Australia). These mice were kept under specific-pathogen-free conditions at the Centenary Institute animal facility with unrestricted access to food and acidified water.

**Bacteria and immunizations.** *M. bovis* BCG (CSL strain) was prepared as previously described (10). Briefly, bacteria were cultured in enriched Bacto Middlebrook 7H9 broth (Difco) containing 0.05% Tween 80 for 7 days at 37°C, 5% CO2 and *M. tuberculosis* H37Rv (ATCC 27294) was grown in Proskauer and Beck liquid medium for 14 days. The bacteria were stored in 30% glycerol at −70°C. Thereafter, bacteria were washed in PBS and sonicated briefly prior to immunizations. BCG was administered by aerosol infection, using a Pari Proneb Turbo nebulizer (Pari Respiratory Equipment Inc.), which produces 3.1-μm-diameter particles at the rate of 6 ml/min. The concentration of BCG in the nebulizing solution was adjusted to deliver approximately 106 mycobacteria to the lungs. Eight milliliters of the BCG (CSL) suspension (~106 CFU/ml of PBS) was aerosolized, and mice were exposed for 30 min. After exposure, mice were kept under UV light for 30 min. The infection dose was confirmed by culture of lung homogenate 2 h after infection. Mice immunized i.v. received an injection of 106 BCG organisms into the lateral tail vein. For the s.c. immunizations, 50 μl of PBS containing 106 bacteria was injected into the right hind footpad of mice by using a 26-gauge needle. Control animals were injected with PBS. Aerosol infection of mice with *M. tuberculosis* was performed as previously described (12).

**Antibodies.** The following monoclonal antibodies (MAbs) were used for flow cytometry: anti-CD44-fluorescein isothiocyanate (FITC) (clone IM7; PharMingen, San Diego, Calif.), anti-CD49d (α4 integrin)-FITC (clone R1-2; PharMingen), anti-CD103 (αε integrin)-FITC (clone 2E7; PharMingen), anti-β7 integrin-phycoerythrin (PE) (clone M293; PharMingen), anti-CD62L-PE (clone MEL-14; PharMingen), anti-CD11c anti-biotin-conjugated anti-CD29 (β1 integrin; clone HA25; PharMingen). Anti-CD4-FITC (clone CT4-FITC) and isotype control antibodies were purchased from Caltag (San Francisco, Calif.). Streptavidin-conjugated with PE (Caltag) was used as a secondary reagent for biotin-labeled antibodies.

**Preparation of single-cell suspensions from lung.** Single-cell suspensions from left lung were prepared as described previously (10). Animals were sacrificed by carbon dioxide narcosis at appropriate time points, and the lung vascular bed was perfused with PBS, 0.02% bovine serum albumin (w/vol), and heparin (20 U/ml; Fisons Pharmaceutical, Sydney, New South Wales, Australia). Lung tissue was minced and then incubated for 90 min at 37°C with shaking in RPMI, supplemented with 50 U of collagenase I (type 4197; Worthington, Freehold, N.J.) per ml and DNase I (13 μg/ml; Boehringer, Mannheim, Germany). After incubation, a single-cell suspension was prepared by removing large aggregates and debris by passage through a 100-μm-pore-size mesh.

**Cell surface staining and flow cytometry.** The detailed procedures for the cell surface staining and flow cytometry analysis of the lung cells have been previously described (4). Briefly, cells were stained for 20 min and washed with 2% bovine serum albumin-0.1% Na3PO4 in PBS. The data were collected using a FACScan (Becton Dickinson Immunocytometry System, San Jose, Calif.) with the CELLQuest program and analyzed after gating on the lymphocyte populations using forward and side scatter. Gating was set on the CD4+ T-cell population based on histograms of the FL3 channel. The staining of samples with isotype control was used as a reference to determine positive and negative populations. Each acquisition file consisted of a minimum of 30,000 events.

**Intracellular IFN-γ staining.** Single-cell suspensions of lung cells were incubated in a 1:100-well plate at 37°C for 1 h to remove adherent cells. Nonadherent cells (106/ml) were then stimulated with plate-bound anti-CD3 MAb (10 μg/ml; PharMingen) for 16 h in complete RPMI (RPMI supplemented with 10% fetal calf serum, 2 mM l-glutamine, 10 mM HEPES, 0.5 μM 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml). Brefeldin A (10 μg/ml; Sigma, St. Louis, Mo.) was added to the cultures for the final 4 h. Cells were washed and surface stained with rat anti-mouse CD4 MAb (Caltag). The cells were fixed with 4% paraformaldehyde for 20 min at room temperature and washed with permeabilization buffer (0.1% saponin in PBS) followed by staining with anti-IFN-γ-FITC (clone AN18) in permeabilization buffer at 4°C for 30 min. Cells were then washed in permeabilization buffer and finally resuspended in FACS buffer (PBS, 2% fetal calf serum) and analyzed on a FACScan flow cytometer.

**Chemotherapy and reinfection.** Twelve weeks after the primary immunizations, mice were treated with ionized zinc (2%; Sigma) in drinking water for 4 weeks to clear bacteria. Following a 2-week interval mice were then reinjected with 105 CFU of BCG by the aerosol route. At defined time points the right lung and spleen were harvested for bacterial counts and the single-cell suspension was prepared from the other lung for cellular analysis.

**Statistical analysis.** Statistical analysis of the results from log-transformed bacterial CFU following infection were carried out using analysis of variance (ANOVA). Fisher’s protected least-significant-difference ANOVA post hoc test was used for pairwise comparison of multigrouped data sets. A difference with a P of <0.05 was considered to be significant.

**RESULTS**

Expansion of activated memory T cells in the lung correlates with the bacterial load during primary infection. To assess the differences in the pulmonary responses following various routes of immunizations, mice were immunized with 103 CFU of BCG by aerosol, i.v., and s.c. routes, and the bacterial load in the lung and the phenotype of infiltrating T cells were analyzed over 12 weeks. Following an aerosol or i.v. infection, significant numbers of bacteria were present in the lung, with the bacterial load peaking in both cases at week 4 (Fig. 1A). Infection by the aerosol route led to a chronic infection in the lung. No bacteria were detected in the lungs following s.c. infection.

Since CD4+ T cells play a dominant role in the immunity to BCG infection, we analyzed the phenotype of CD4+ T cells in the lung. The expansion of the activated memory CD4+ T lymphocyte population, as determined by the expression of
CD44 and CD62L (Fig. 1B) after gating on CD4+ T cells, correlated with the number of bacteria in the lung. The number of CD44hi CD62Llow CD4+ T cells peaked at 8 weeks postinfection and was maximal and sustained following aerosol infection (Fig. 1C). These T cells were also CD45RBlow and positive for CD69, an early activation marker (data not shown).

Integrin expression on CD4+ T cells during primary infection. In order to determine the pattern of T-cell recruitment to the lung during the primary response, the expression of the integrins β7 and α4 and β7 and α4 on pulmonary CD4+ T cells was analyzed. Following aerosol infection there was an increase in the expression of the integrins α4 and β7 on CD4+ T cells (Fig. 2A). There was no increase in the proportion of CD4+ T cells expressing α4β7 after BCG infection (Fig. 2A). There was also an increase in the number of α4β7+ CD4+ T cells in the lungs following i.v. BCG infection, but not following s.c. infection. These T cells also peaked at 8 weeks (Fig. 2B).

Activated memory cells expand rapidly following reinfection. Activated memory T cells induced during primary infection. Following antibiotic treatment to clear the pulmonary infection, mice were challenged with BCG by the aerosol route and the pattern of T-cell recruitment in the lung was examined. There was a rapid increase in the number of CD44hi CD62Llow CD4+ T cells in infected control mice and 8 weeks after aerosol BCG infection. Results correspond to groups of three animals and are representative of two separate experiments. The differences between mice immunized by the aerosol route and i.v. or s.c. routes were tested by ANOVA (*, *P < 0.05).
munized by aerosol, i.v., and s.c. routes. Furthermore the kinetics for the emergence of this population were also similar in the three immunized groups.

**β** ⎯ **CD4** T cells produce IFN-γ during reinfection. Since immunity to mycobacterial infection is characterized by a Th1 response and IFN-γ is essential for protective immunity, we examined IFN-γ production by intracytoplasmic staining of **β** T cells and **β** T cells at 4 weeks after challenge with BCG. The major IFN-γ production occurred in the **β** CD4 T-cell population, of which approximately 10% were cytokine producers (Table 1). IFN-γ cytokine production was similar in mice immunized by the three routes.

**Integrin expression on CD4 T cells following M. tuberculosis challenge.** The effect of different routes of immunization on integrin expression on CD4 T cells in the lung was analyzed following reinfection with *M. tuberculosis*. The dual expression of the memory cell marker CD44 and integrin **β** showed that most of the CD44 CD4 T cells were **β** (Fig. 4A). The numbers of CD44 **β** CD4 T cells (Fig. 4B) and **β** CD4 T cells (Fig. 4D) in the lung increased to similar extents in mice originally immunized by aerosol, i.v., and s.c. routes (Fig. 4B).

**Protective efficacy of immunizations by different routes.** In order to test the protective efficacy of prior BCG immunization by the three different routes, mice were treated with antibiotics prior to rechallenge by the aerosol route. This resulted in clearance of infection in i.v. and s.c. groups and reduction to 10 CFU of bacteria per lung in the aerosol group (data not shown). Following reinfection with aerosol BCG, immunization by all three routes resulted in significant and similar reductions in the number of bacteria in the lungs compared to unimmunized mice. The differences were apparent at 2 weeks and were sustained at 4 weeks postreinfection (Fig. 5A). We repeated the experiment with groups of 10 mice and found a similar level of protection both in the lung (data not shown) and spleen (Fig. 5B) between mice immunized by all different routes at 4 weeks postreinfection.

In order to test the protective efficacy of prior BCG immunization by the three different routes against aerosol *M. tuberculosis* infection, immunized mice were treated with antibiotics and then challenged with aerosol *M. tuberculosis*. Four weeks after infection the bacterial loads in the lungs and spleen were enumerated. All immunized mice had significant and similar reductions in the bacterial loads in both the lung (Fig. 6A) and the spleen (Fig. 6B). There were no differences in the bacterial load in the lung following s.c. immunization (4.5 log10 CFU), i.v. immunization (4.49 log10 CFU), and aerosol immunization (4.47 log10 CFU). Similarly, the protection level in the spleen was also similar in all immunized mice, regardless of the route of immunization.

**DISCUSSION**

This study adds to our understanding of the mechanism of recruitment of CD4 T cells to the lungs during primary immunization with BCG and subsequent aerosol challenge with mycobacteria. During the primary response the number of activated memory CD4 T cells in the lung correlated with the
bacterial load, which varied for the three routes of immunization. Although the bacterial load was maximal in the aerosol group, similar patterns of expansion of lung activated memory CD4^+ T cells and integrin expression on CD4^+ T cells occurred in both aerosol and i.v. immunized mice. The majority of the infiltrating CD4^+ T cells expressed the α4β1 integrin. Following subsequent challenge, however, all immunized mice had similar rapid expansion of α4β1^+ CD4^+T cells within the lungs (Fig. 5A). Interestingly although there was no expansion of activated memory, α4β1^+ T cells in the lungs during the primary response in the s.c. immunized mice, aerosol reinfection resulted in equivalent recruitment of these cells to the lungs in these mice compared to the aerosol or i.v. immunized groups. These data suggest that T-cell homing to the inflamed peripheral lung resembles the interactions that govern T-cell recruitment to systemic inflammatory sites, and therefore similar numbers of protective T cells are recruited to the inflamed lung during *M. tuberculosis* infection, regardless of the route of primary BCG immunization. Immunization by the three routes resulted in significant protection both in the lungs and spleen.

The binding of α4β1 integrin (VLA-4) on T lymphocytes to VCAM-1 on activated endothelia facilitates the recruitment of T cells to sites of inflammation (9). VLA-4 is upregulated on T cells 7 to 10 days after activation (16) and persists on memory T cells (30). During *M. tuberculosis* infection of the lung, recruitment of both CD4^+ and CD8^+ α4β1 T cells peaks at 8 weeks (12). The addressin VCAM-1 appears on pulmonary endothelia 2 weeks following *M. tuberculosis* infection and persists at high levels to 12 weeks (12). VCAM-1 was also upregulated on pulmonary endothelia during aerosol BCG infection (data not shown). The mucosal addressin MAdCAM-1 was not
apparent on vascular endothelia during either M. tuberculosis (12) or BCG infections (data not shown), and T cells bearing /52 were not a significant component of cellular influx. The role of /32 T cells in controlling M. tuberculosis infection was confirmed by exacerbation of infection and dysregulation of the inflammatory response when recruitment of /32 T cells was blocked (12). In the present study, BCG immunization by all three routes primed populations of mycobacterium-specific T cells. These were efficiently recruited to the lung following subsequent aerosol BCG or M. tuberculosis infection, indicating that the peripheral lung acts more like an inflammatory site, rather than a mucosal site, during effector immune responses.

Previous attempts to assess the relationship between the protective efficacy of BCG immunization and the route of immunization have led to contradictory conclusions. Initial studies by Barclay et al. (3) showed a greater degree of protection against an aerosol challenge with M. tuberculosis in monkeys that had received BCG by the aerosol or i.v. routes, compared to those immunized s.c. Later, Lefford (23) found that the pulmonary mycobacterial load on M. tuberculosis challenge was significantly lower in mice immunized with BCG by the aerosol route than in those immunized by i.v. or s.c. routes. Further studies in mice (27) and in guinea pigs (19, 20) suggested that aerogenic BCG immunization imparted more protection than peripheral s.c. immunization. In adoptive transfer experiments, however, Orme and Collins (25) demonstrated that aerogenic immunization with BCG offered no protective advantage over s.c. immunization in mice. This finding has also been supported in other animal models of mycobacterial infections, such as possums (1) and cattle (6).

None of these studies examined the immunological basis for
the apparent differences or the pattern of T-cell recruitment to the lung following the different routes of immunization. It is clear from the present study that the number of mycobacteria in the lung following the different routes of BCG immunization is probably due to the infection. Therefore, the equivalent protective efficacy for the three routes of BCG immunizations is probably due to the similar process and speed of lymphocyte recruitment.

Identifying the homing signals that govern the recruitment of effector T cells to the lung is important for the delivery of future anti-TB vaccines, as the site of initial priming influences the circulation pattern for T cells. It is clear from the present study that the signals regulating T-cell homing to the peripheral lung and systemic inflammatory sites are similar, and therefore peripheral s.c. immunization with BCG or other antitubercular vaccines should be as effective as immunization by the aerosol route.

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