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

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Received 7 September 2001/Returned for modification 1 November 2001/Accepted 21 November 2001

*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 predominantly through the interaction of α₄β₇ 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 CD44⁺ 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 α₄β₇ integrin. On subsequent exposure to aerosol BCG rapid expansion of gamma interferon-secreting α₄β₇⁺ CD4⁺ T cells occurred to the same extent in all immunized mice, regardless of the route of immunization. Similar expansion of α₄β₇⁺ 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 aerosgenic vaccination with BCG offered no immunological 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

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influence the immune response to *M. tuberculosis* in the lung. Immunization by systemic routes stimulates effector memory T cells expressing the integrin αβ7 (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 αβ7, 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 αβ7 integrin and not the αβ1 integrin (12). Further, there was increased expression of the VCAM-1, but not MAdCAM-1, on pulmonary endothelia during *M. tuberculosis* infection, suggesting that αβ7+ CD4+ T cells were sufficient for protective immunity. Therefore, we hypothesized that activation of αβ7+ 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 αβ7 integrin in the lung after the primary immunizations, there was a similar degree of expansion of αβ1+ 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 (C5L 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-phosphate-buffered saline (PBS) 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-m diameter particles at the rate of 6 ml/min. 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-m diameter particles at the rate of 6 ml/min. By contrast, antigen encounter by mucosal routes leads to the development of memory T cells expressing αβ7, 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 αβ7 integrin and not the αβ1 integrin (12). Further, there was increased expression of the VCAM-1, but not MAdCAM-1, on pulmonary endothelia during *M. tuberculosis* infection, suggesting that αβ7+ CD4+ T cells were sufficient for protective immunity. Therefore, we hypothesized that activation of αβ7+ 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.

**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 10^3 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 gaging on CD4⁺ T cells, correlated with the number of bacteria in the lung. The number of CD44⁺⁺⁺ CD62L⁺⁻ CD4⁺ T cells peaked at 8 weeks postinfection and was maximal and sustained following aerosol infection (Fig. 1C). These T cells were also CD45RB⁺⁻ 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 after s.c. infection. These T cells also peaked at 8 weeks (Fig. 2B).

**Activated memory cells expand rapidly following reinfection.** Acquired immunity on reexposure to mycobacterial infection is dependent on the rapid development of CD4⁺⁺ 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 CD44⁺⁺⁺ CD62L⁺⁻ CD4⁺⁺ T cells in the lung. 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).
mumized 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.

β1+ 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 β1+ and β7+ CD4+ T cells at 4 weeks after challenge with BCG. The major IFN-γ production occurred in the β1+ 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 β1 showed that most of the CD44hi CD4+ T cells were β1+ (Fig. 4A). The numbers of CD44hi β1+ CD4+ T cells (Fig. 4B) and αβ1+ 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), i.v. immunization (4.49 log10), and aerosol immunization (4.47 log10). 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 \(\alpha_4\beta_1\) integrin. Following subsequent challenge, however, all immunized mice had similar rapid expansion of \(\alpha_4\beta_1\) CD44\(^hi\) activated memory T cells within the lungs (Fig. 5A). Interestingly although there was no expansion of activated memory, \(\alpha_4\beta_1\) 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 \textit{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 \(\alpha_4\beta_1\) 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 \textit{M. tuberculosis} infection of the lung, recruitment of both CD4\(^+\) and CD8\(^+\) \(\alpha_4\beta_1\) T cells peaks at 8 weeks (12). The addressin VCAM-1 appears on pulmonary endothelia 2 weeks following \textit{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 CD4/CD8 markers were not a significant component of cellular influx. The role of CD4/CD8+ T cells in controlling *M. tuberculosis* infection was confirmed by exacerbation of infection and dysregulation of the inflammatory response when recruitment of CD4/CD8+ 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 reaching the lungs determines the pulmonary T-cell infiltration during initial immunizations. Subsequent aerosol challenge results in equivalent recruitment of activated memory $\alpha_4\beta_7$ $\text{CD}^4$ T cells regardless of the route of BCG immunization. This cellular influx was more rapid in immunized mice and was associated with an earlier and smaller peak of pulmonary 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 antitymocobacterial vaccines should be as effective as immunization by the aerosol route.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia. The support of the NSW Health Department through its research and development infrastructure grants program is gratefully acknowledged. U. Palendira and C. G. Feng are recipients of Australian Postgraduate Awards. U. Palendira is also supported by the Cooperative Research Center for Vaccine Technology.

We thank Jason Compton for technical assistance and H. Briscoe and B. Saunders for helpful discussions.

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Editor: D. L. Burns