

Murine model of BCG lung infection: Dynamics of lymphocyte subpopulations in lung interstitium and tracheal lymph nodes

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C57Bl/6 female mice were infected with an intrapulmonary dose of 2.5×10^4 BCG (*Mycobacterium bovis* Bacillus Calmette-Guerin). Lymphocyte populations in lung interstitium and lung-associated tracheal lymph nodes (LN) were examined at 1, 2, 4, 5, 6, 8 and 12 weeks after infection. BCG load in lungs peaked between 4–6 weeks post-infection and declined to very low levels by the 12th week of infection. Lung leukocytes were obtained over the course of infection by enzyme digestion of lung tissue followed by centrifugation over Percoll discontinuous density gradients. By 4 to 6 weeks after infection, numbers of lung leukocytes had more than doubled but the proportions of lymphocytes (about 70%), macrophages (about 18%) and granulocytes (about 12%) remained essentially unaltered. Flow cytometric studies indicated: (i) the total number of CD3⁺ T cells in lungs increased by 3-fold relative to uninfected controls at 5 to 6 weeks post-infection, but the relative proportions of CD4 and CD8 cells within the T cell compartment remained unaltered; (ii) relative proportion of NK cells in lungs declined by 30% but the total number of NK cells (NK1.1⁺) per lung increased by about 50%, 5–6 weeks post infection; (iii) tracheal LN underwent marked increase in size and cell recoveries (6–10-fold increase) beginning 4 weeks after infection. While both T and B cells contributed to the increase in cell recoveries from infected tracheal LNs, the T/B ratio declined significantly but CD4/CD8 ratio remained unaltered. In control mice, IFN γ producing non-T cells outnumbered T cells producing IFN γ . However, as the adaptive response to infection evolves, marked increase occur in the number of IFN γ producing T cells, but not NK cells in the lungs. Thus, T cells are the primary cell type responsible for the adaptive IFN γ response to pulmonary BCG infection. Few T cells in tracheal LN of BCG infected mice produce IFN γ , suggesting that maturational changes associated with migration to the lungs or residence in the lungs enhance the capability of some T cells to produce this cytokine.

[Saxena R K, Weissman D, Simpson J and Lewis D M 2002 Murine model of BCG lung infection: Dynamics of lymphocyte subpopulations in lung interstitium and tracheal lymph nodes; *J. Biosci.* **27** 143–153]

1. Introduction

One third of all human beings are estimated to be infected with *Mycobacterium tuberculosis*, which makes tuberculosis one of the most serious global health problems. Three million people die of TB and 8 million fresh cases are detected each year (Bleed *et al* 2000). BCG (*Myco-*

bacterium bovis Bacillus Calmette-Guerin) which is the only vaccine available against tuberculosis, has an efficacy ranging from 0% to 80% (Fine 1989; Orme 1999). A proper understanding of immune response to *M. tuberculosis* will pave the way for developing more effective vaccines against tuberculosis. Immune response to *M. tuberculosis* in the mouse model has been studied

Keywords. BCG; interferon response; lung infection; lymph nodes; Murine model; NK cells; T cells; tuberculosis

(Huygen *et al* 1992; Andersen 1997; Ibsen *et al* 1997). Systemic and lung infections with mycobacteria may induce qualitatively different types of immune responses. Mice were reported to be a hundred-fold more susceptible to infection initiated in lungs than the infection initiated systemically (North 1995). The primary portal of entry of *M. tuberculosis* is the lung. It is therefore important to understand the nature of immune responses to mycobacterial infection in lungs. BCG lung infection in mice resembles human infection in as much as the infection is effectively contained in both cases. Some information about the immune response to BCG lung infection in mice is available (Wakeham *et al* 1998; Xing *et al* 1998; Fulton *et al* 2000). Infection peaks at 4 to 6 weeks after intratracheal (IT) deposition of BCG and there is an associated surge in T cells in the lung interstitium (Fulton *et al* 2000). Broncho-alveolar lavage contains increased levels of cytokines associated with T-helper (Th)1-type responses such as IFN γ , IL12 and TNF α (Wakeham *et al* 1998). Mouse models using immunodeficient and knockout mice have demonstrated a crucial role of type I cytokine response, especially IFN γ , in protective immunity to *M. tuberculosis* (Flynn *et al* 1993; North and Izzo 1993; Cooper *et al* 1993, 1997; Wakeham *et al* 1998). T cells as well as NK cells can produce IFN γ . The kinetics of activation and accumulation of these cells in lungs of infected mice is not clearly understood. Moreover the relative contribution of these lymphocyte subsets to IFN γ secretion is not known.

The over-arching hypothesis for the current study is that Th1 responses, particularly IFN γ responses, play a key role in clearance of mycobacterial infection from the lungs. Two types of lymphocytes i.e. T cells and NK cells can produce IFN γ . While the crucial role of IFN γ in protective immunity to mycobacterial infections has been demonstrated, relative contribution of T and NK cells to the local secretion of IFN γ in lungs infected with mycobacteria is not known. In the present study, we have documented the kinetics of changes in various lymphocyte subpopulations after murine pulmonary BCG infection. Relative contribution of T and NK cells in producing IFN γ has also been analysed. Our results show that both T and NK cells contribute to local IFN γ production in control and BCG infected mouse lungs. In BCG infected mice however, the augmented IFN γ response is essentially due to the expansion of IFN γ producing T cell subsets.

2. Materials and methods

2.1 Mice

C57Bl/6 female mice 12 to 15 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME, USA and

used between 18 and 30 weeks of age. Mice were maintained in filter-topped cages provided with HEPA-filtered air in the animal facilities at NIOSH. All studies were reviewed and approved by the NIOSH Institutional Animal Care and Use Committee.

2.2 Reagents

Percoll, phorbol 12-myristate 13-acetate (PMA), ionomycin, collagenase (cat No. C5138), DNase (cat. No. D4263), hyaluronidase (cat No. H3506), elastase (cat No. E0258), goat-anti-mouse Ig-FITC (specific for Fab), and RPMI 1640 culture medium were bought from Sigma, St. Louis, MO, USA. Monoclonal antibodies (Mabs) labelled with FITC or PE [anti-CD3 (clone 17A2); anti-NK1.1 (clone PK136); anti-CD4 (clone GK1.5); anti-CD8 (clone 53-6.6); anti-CD16/32 (Fc-block, clone 2.4G2); anti-mouse IFN γ (clone XMG1.2)] and their respective isotypic controls were obtained from Pharmingen (Pharmingen/Becton Dickinson, San Diego, CA, USA). Middlebrook culture medium and supplements were obtained from Becton Dickinson Microbiology Systems, Sparks, MD, USA.

2.3 Intrapulmonary instillation of BCG

A seed culture of BCG (*M. bovis* Pasteur, TMCC No. 1011) was kindly provided by Prof. Ian Orme of the Microbiology Department, Colorado State University, Fort Collins, CO, USA. BCG was grown in Middlebrook culture medium with OADC supplement and Tween. Viable BCG were counted by plating bacterial suspensions at different dilutions on Middlebrook agar plates and counting colonies after two weeks. Intrapulmonary instillation of BCG suspension (50 μ l bacterial suspension containing 2.5×10^4 bacteria/mouse) or PBS was carried out as described by Keane-Myers *et al* (1998).

2.4 Tissue processing

Mice were sacrificed by pentobarbital overdose. Spleen and liver were homogenized in 10 ml PBS using a Polytron homogenizer. Aliquots (100 μ l) of neat or appropriately diluted homogenates were plated on Middlebrook Agar plates. BCG colonies were counted two weeks later. In order to minimize contamination of lung interstitial leukocyte preparations by blood derived cells, lungs were perfused with PBS via the right atrium before their removal. Isolation of lung interstitial cells was done by

enzymatic digestion of lung tissue, mechanical dispersion, and separation on a discontinuous Percoll gradient essentially as described (Wakeham *et al* 1998). Briefly, lung tissue was chopped into small pieces (1–2 mm) and incubated in a solution containing collagenase (150 U/ml), DNase (20 Kunitz/ml), hyaluronidase (35 U/ml) and elastase (0.2 U/ml) in PBS, for 90 min on a rocking platform at 37°C. Digested lung tissue was grounded through a stainless steel mesh and the resulting suspension filtered through 100-micron filter to remove tissue debris. Leukocytes were isolated by discontinuous Percoll gradient centrifugation (Wakham *et al* 1998). Differential cell counts were performed using cytopsin preparations stained with May-Grunwald-Giemsa. For each cell preparation, at least 200 cells were counted. The right tracheal lymph node (LN) known to receive drainage from the lung, was removed from the superior right side of the mediastinum cavity and teased in PBS to obtain cells.

2.5 Flow cytometric analysis

Cells derived from lungs and tracheal LN were stained with monoclonal antibodies against several membrane markers as well as against IFN γ to detect cytoplasmic IFN γ . Staining protocols suggested by the manufacturer (BD Pharmingen, San Diego, CA, USA) were followed. Cells were analysed on a Becton Dickinson FACS Calibur flow cytometer. Cells stained with isotypic control antibodies were used to demarcate the lymphocyte window as well as to set gates for discrimination between cells with or without specific stain. The gates would have included all types of lymphocytes.

2.6 Serum anti-BCG antibody titers

Anti-BCG antibody concentrations in mouse sera were determined by ELISA as described before (Udaykumar *et al* 1991). Briefly, ELISA plate wells were coated with BCG sonicate antigen (1 μ g in 50 μ l/well in 0.2 M bicarbonate buffer, pH 9.6, overnight at room temperature) and blocked with milk proteins (1.0% milk powder in PBS). Reactivity was studied with various diluted mouse sera (50 μ l/well, overnight incubation at 4°C). Bound antibody was detected using a goat anti-mouse-Ig-alkaline phosphatase detecting antibody followed by the chromogenic substrate *p*-nitrophenyl phosphate. Relative antibody concentrations are expressed as colour development measured as absorbance at 405 nm.

2.7 Statistical analysis

Unless specified otherwise, data from at least five control and 5 BCG infected mice were compared at different time points in all experiments. Each experiment was repeated at least three times with reproducible results. Representative data from each experiment has been

shown. Two-way comparisons were performed using Student's *t*-test. Multiple comparisons were performed by analysis of variance and an appropriate multiple comparison procedure. Comparisons were made using computer software (Sigmastat, SPSS, Chicago, IL, USA). Comparisons were considered significantly different at a level of $P \leq 0.05$.

3. Results

At least three experiments were done to confirm each result. Only representative data have been shown, based on an experiment utilizing 70 mice. Ten mice (5 control and 5 infected mice) were sacrificed at each of the seven time points shown. Representative data for antibody titres in sera samples from four infected mice at different times after the initiation of the lung infection, are shown in figure 7.

3.1 Course of BCG infection in C57Bl/6 mice

Results in figure 1 show BCG counts in lung, spleen and liver of BCG-instilled mice at various time points after

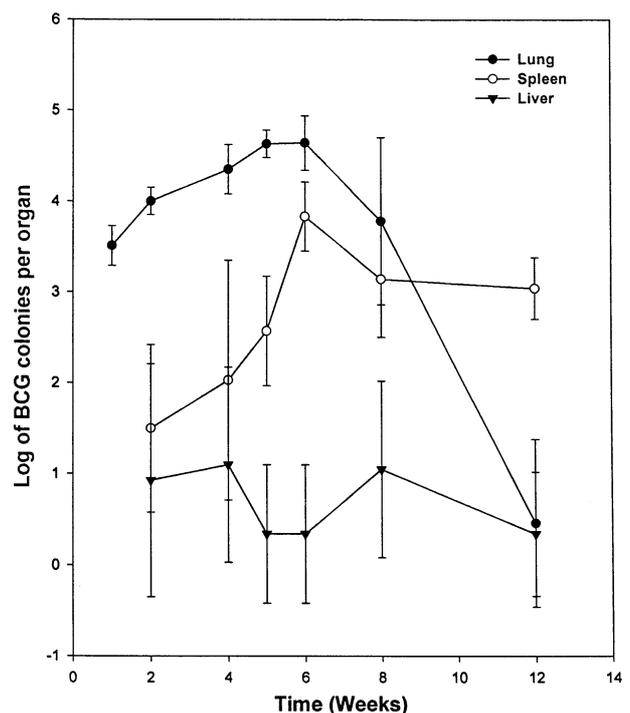


Figure 1. Bacterial load in lungs, spleens and livers of C57Bl/6 with intrapulmonary instillation of BCG. BCG was instilled in lungs of C57Bl/6 female mice (2.5×10^4 bacteria in 50 μ l PBS to each mouse). At various given time periods after the initiation of the infection, BCG load in lungs, spleens and livers from infected mice was determined as described in §2. Bacterial loads in spleen and liver after one week of infection were too low to be included. Each value represents a mean \pm SD of five observations.

infection. One week after infection, BCG recovery from lungs averaged 10% of the instilled inocula. BCG load in lungs increased and peaked between 4 to 6 weeks, after which it declined. Highest average bacterial load (4.3×10^4 /lung) was attained six weeks after infection. By the 12th week after infection, BCG were essentially cleared from lungs. Parallel assessment of BCG load in spleen indicates that one week after infection, spleens were devoid of any bacterial load. Few BCG colonies could be detected in spleens two weeks after infection, and the bacterial load further increased till 6th week, after which it appeared to plateau. Significant metastasis of BCG to liver was not observed, as the average BCG load remained below 100 colonies per liver at all time points.

3.2 Effect of BCG lung infection on the recovery of lung interstitial leukocytes

Interstitial lung leukocytes were obtained by enzyme digestion of lung tissue followed by discontinuous Percoll gradient centrifugation. Using this technique, average leukocyte recovery in control groups of mice varied between 1.2 and 3.0×10^6 /lung at different time points (figure 2). At each time point the recovery of lung interstitial leukocytes from BCG instilled mice was compared to the corresponding recoveries from PBS instilled control mice. Results in figure 2 indicate that leukocyte recovery from lungs of infected mice was significantly greater than the control recoveries at 5, 6, and 8 weeks after infection (figure 2, lower panel). Maximum increases of about

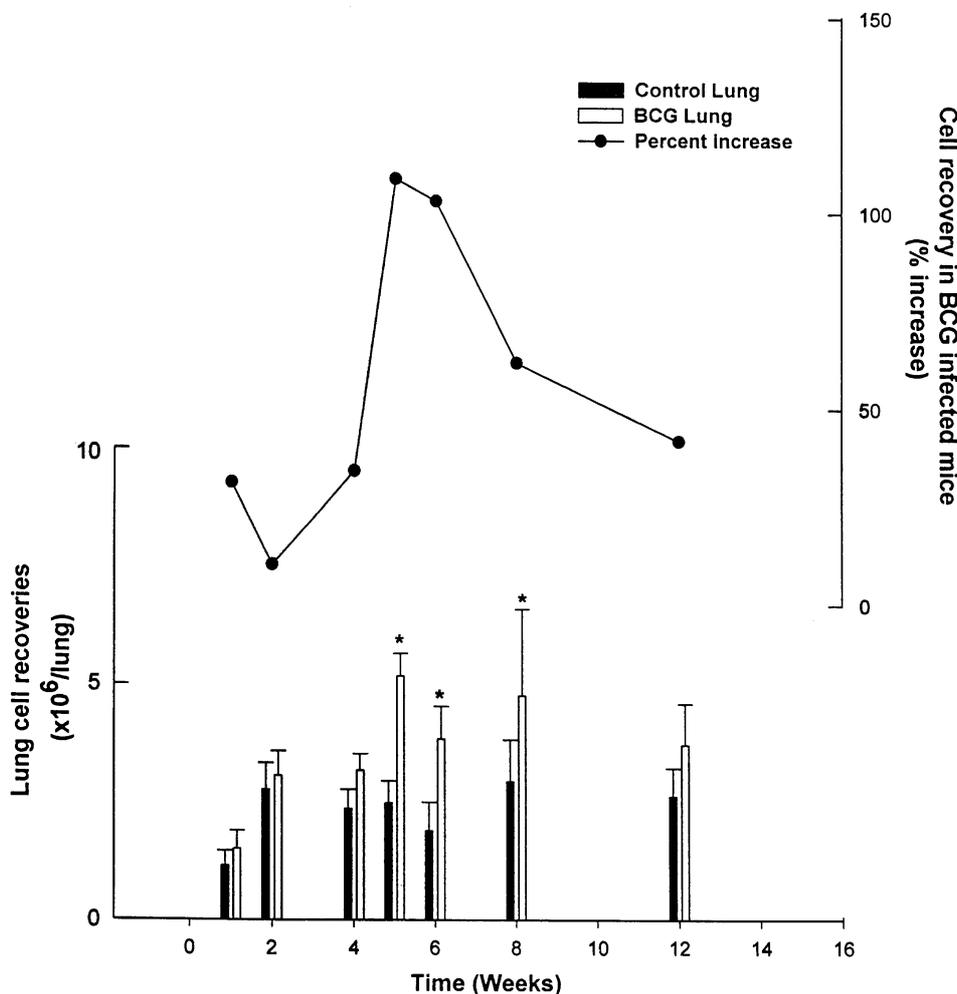


Figure 2. Time kinetics of leukocyte recoveries from lungs of control (PBS instilled) and infected (BCG instilled) C57Bl/6 mice. Y axis scale on the left corresponds to the histograms in the lower part of the figure and the Y axis scale on the right is for the curve shown in the upper part of the figure. Each value of cell recovery is a mean \pm SD of five observations. *BCG group different from control group, unpaired *t*-test, $P < 0.05$.

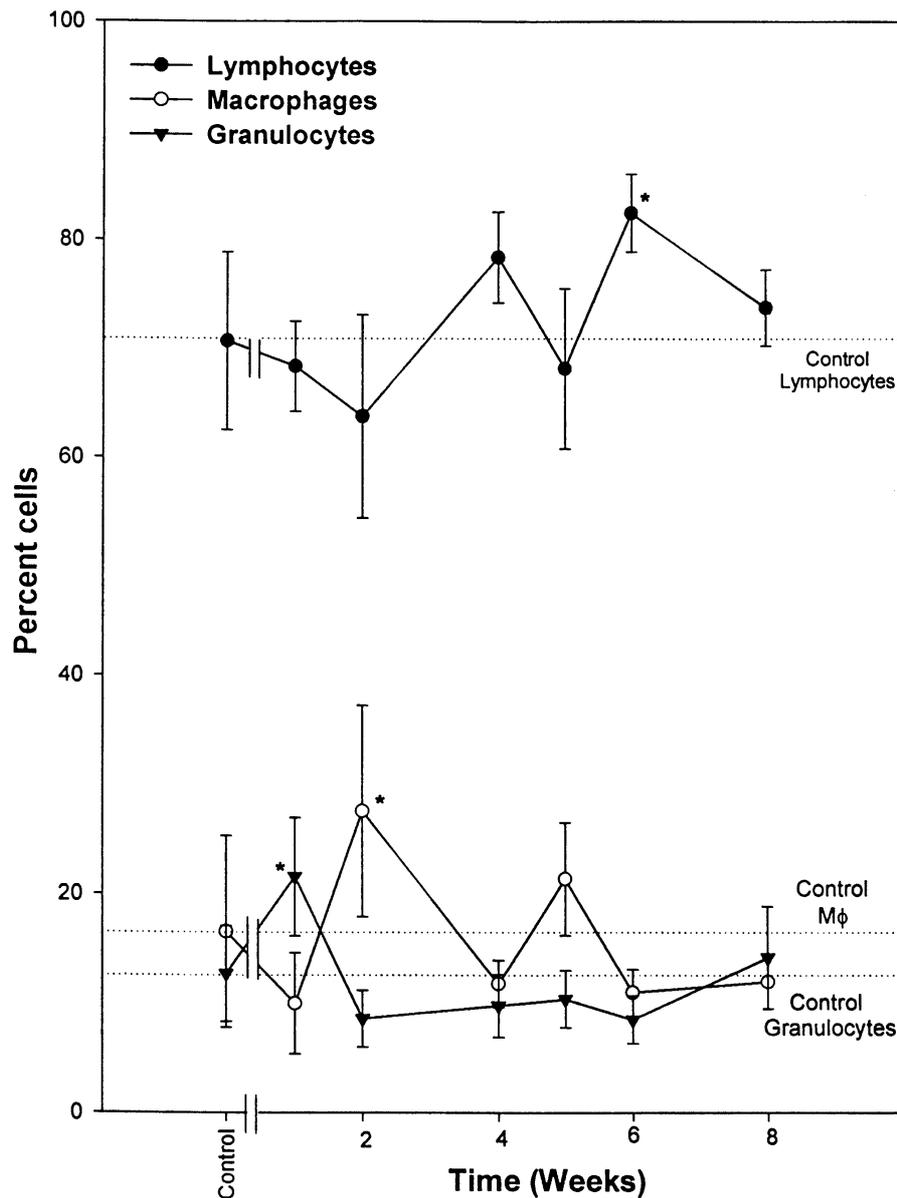


Figure 3. Proportions of lymphocytes, macrophages and granulocytes in leukocyte preparations derived from lungs of control (PBS instilled) and infected (BCG instilled) mice. Values of differential counts represent mean \pm SD of 5 observations. Horizontal dotted lines represent mean \pm SD of 30 observations in non-infected control group. *Difference of BCG group from control group by Dunnett's multiple comparison test, $P < 0.05$.

100% were seen on the 5th and 6th weeks post-infection (figure 2, upper panel). Differential leukocyte count was done on cell preparations derived from lungs. Typically, lymphocytes, macrophages and granulocytes comprised $70.7 \pm 8.2\%$, $16.6 \pm 8.8\%$ and $12.7 \pm 4.4\%$ respectively of the total lung leukocytes derived from control mice (dotted lines in figure 3). The proportions of lymphocytes, macrophages and granulocytes in lungs of mice infected

with BCG at different intervals after infection are shown in figure 3. In general, no large fluctuations were noted in the relative proportions of lymphocytes, macrophages, and granulocytes over the course of BCG infection. However, a statistically significant ($P < 0.05$) increase in the proportions of neutrophils, macrophages and lymphocytes relative to control was observed at the 1st, 2nd and 6th week points respectively (figure 3).

3.3 Phenotypic make up of lymphocytes derived from lungs of control and BCG infected mice

Flow cytometric analysis was used to enumerate lymphocyte subpopulations in lungs at each time point. In all phenotypic studies, a lymphocyte gate was set on the forward/side scatter plot. Cells were stained and analysed for the expression of CD3, CD4, CD8, NK1.1, *gd* TCR and surface Ig markers. T cells (CD3⁺) as percentage of all lung lymphocytes from BCG or PBS (control) instilled mice, are shown in the bottom panel of figure 4. These results indicate that the absolute numbers of T cell proportions increased significantly in BCG infected mice especially at the 5th week time point, when the increase in T cell proportion was about 50% (top panel, figure 4). Similar data for NK cell proportions are given in figure 5. Proportions of NK cells declined by 30% relative to control ($P < 0.05$) at the 4th, 5th and 6th week points, which corresponded with an increase in relative proportion of T cells at these time points. T cells thus increased and NK cell decreased in BCG infected lungs in terms of

percentages of all lung lymphocytes. Figure 2 however shows that the total lung cell recovery doubled in BCG infected lungs at the peak of infection. Knowing the total cell recoveries and the proportion of T and NK cells, absolute numbers of T and NK cells in control and BCG infected lungs could be computed. Absolute numbers of both T and NK cells increased in BCG infected lungs, though the increase was much larger for T cells (about 3-fold) than for NK cells (about 50% increase).

Proportions of CD4⁺ and CD8⁺ T cells were also determined in lung cells from control and BCG infected mice 5, 6, and 8 weeks post infection. The average CD4/CD8 ratio in lungs of control mice was 1.2 ± 0.21 (mean \pm SD of 20 determinations, range 0.97 to 1.90). This ratio was not significantly altered at any time point in BCG infected mice and was 1.22 ± 0.08 , 1.22 ± 0.13 and 1.27 ± 0.03 respectively at 5, 6 and 8 weeks post infection. Relative proportions of *gd* T cells in lung-derived lymphocytes was $< 2\%$ and remained unaltered in BCG infected mice examined 5 weeks post infection (results not shown). Proportion of surface Ig⁺ B cells in lung

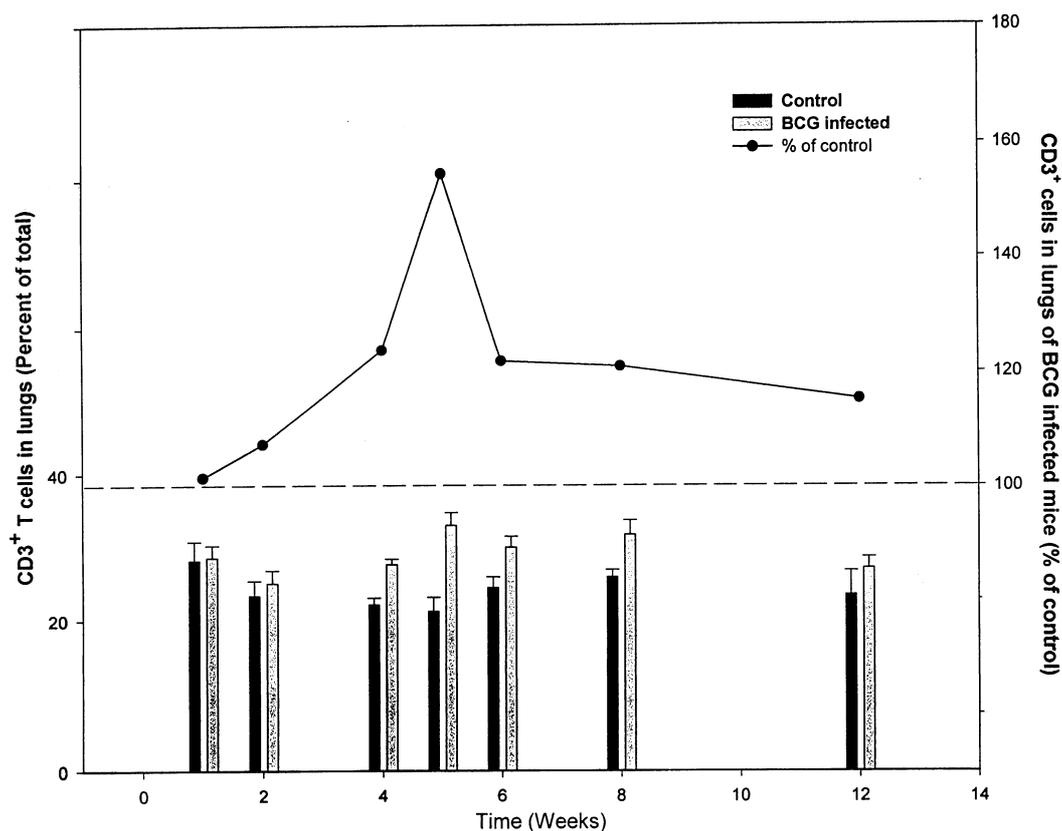


Figure 4. Time kinetics of changes in proportions of CD3⁺ lymphocytes in lungs of control (PBS instilled) and infected (BCG instilled) mice. Lower panel (Y axis scale on the left) denotes the percentage of CD3⁺ cells in lung cell preparations from control and BCG infected mice. Upper panel (Y axis scale on the right) shows the percent change in the proportions of T cells in lungs of BCG infected mice. All values in lower panel represent mean \pm SD of 5 observations.

lymphocytes in control and BCG infected mice (6 week point) were $44.4 \pm 2.36\%$ and $45.1 \pm 1.97\%$ respectively, indicating that the relative proportions of B cells were not changed in the lungs of BCG infected mice.

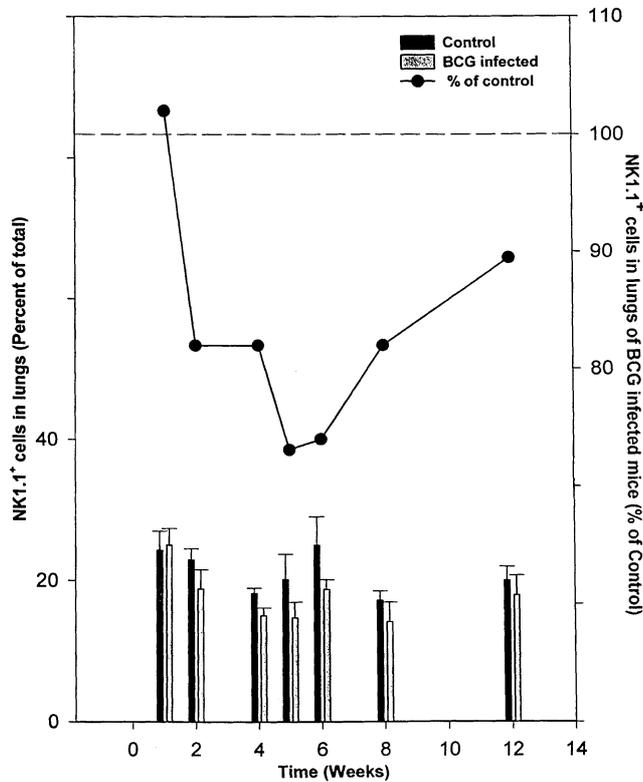


Figure 5. Time kinetics of changes in proportions of NK1.1⁺ lymphocytes in lungs of control (PBS instilled) and infected (BCG instilled) mice. Lower panel (Y axis scale on the left) denotes the percentage of NK1.1⁺ cells in lung cell preparations from control and BCG infected mice. Upper panel (Y axis scale on the right) shows the percent change in the proportions of NK cells in lungs of BCG infected mice. All values in lower panel represent mean \pm SD of 5 observations.

3.4 Lymphocyte subpopulations in tracheal LN from control and BCG infected mice

Recoveries of cells from tracheal LN in control and BCG infected mice are shown in table 1. Average lymphocyte recoveries from tracheal LN of uninfected mice was $1.29 \pm 0.54 \times 10^6$ which increased by 6- to 12-fold in BCG infected mice (table 1). Both T and B cells contributed to increased cell recovery, though the increase in B cell numbers (> 12-fold) was relatively greater than the increase in T cells (7-fold). NK cells and *gd* T cells comprised less than 1% of lymph node cells and no significant change in the proportion of these cells occurred in BCG infected mice (results not shown).

3.5 IFN γ production by lung and tracheal LN cells at the peak of BCG infection

Both T and NK cells have the capacity to secrete IFN γ . We examined the proportion of T cells in lung interstitium and tracheal lymph nodes, producing IFN γ at the peak of BCG infection. Results of a representative experiment of two colour flow cytometry with intercellular cytoplasmic staining for IFN γ and surface staining for CD3 marker, are given in figure 6 (upper panel). These results show that 11.67% of the total lymphocytes in lung interstitium were T cells with intercellular IFN γ whereas this percentage was 2.44% for control lungs. There was thus a marked increase (4-5-fold) in T cells making IFN γ in BCG infected lungs. In contrast to lung cells from control and BCG infected mice, tracheal LN cells from control as well as BCG infected mice had few IFN γ producing cells (figure 6, bottom panel). The proportion of tracheal LN cells, which stained for cytoplasmic IFN γ in all such experiments remained less than 1%.

Significant numbers (4-8% of lung lymphocytes) of non-T cells (CD3 negative) produced IFN γ in control lungs, and this percentage remained unchanged in BCG infected lungs (figure 6, upper panel). Non-T cells (CD3⁻)

Table 1. Cell recoveries from lung associated tracheal lymph nodes from control and BCG infected mice.

Treatment group	Duration of infection	Cells/lymph node ($\times 10^6$)				
		Total	CD3 ⁺	CD4 ⁺	CD8 ⁺	sIg ⁺
Control	None	1.29 ± 0.54	0.64 ± 0.24	0.33 ± 0.12	0.30 ± 0.13	0.57 ± 0.29
BCG infected	4 weeks	$8.72 \pm 4.15^*$	$3.39 \pm 1.66^*$	ND	ND	$4.65 \pm 2.26^*$
BCG infected	8 weeks	$12.82 \pm 6.46^*$	$4.63 \pm 1.73^*$	$2.31 \pm 0.78^*$	$2.31 \pm 0.83^*$	$6.80 \pm 4.05^*$
BCG infected	12 weeks	$12.02 \pm 8.02^*$	$4.38 \pm 3.07^*$	$2.21 \pm 1.48^*$	$2.17 \pm 0.59^*$	$7.11 \pm 4.75^*$

Lymph node cells were obtained from control (PBS instilled) and infected (BCG instilled) mice and analysed for various surface markers. Each value is a mean \pm SD of 5 to 7 observations. Statistical difference between control and infected groups were assessed by analysis of variance and Dunnett's multiple variance procedure (* $P < 0.05$). ND, Not determined.

with cytoplasmic IFN γ are likely to be NK cells. To confirm this, we stained lung lymphocytes derived from 5 week BCG infected mice with two combinations of antibodies, (i) membrane staining with anti-NK1.1 and

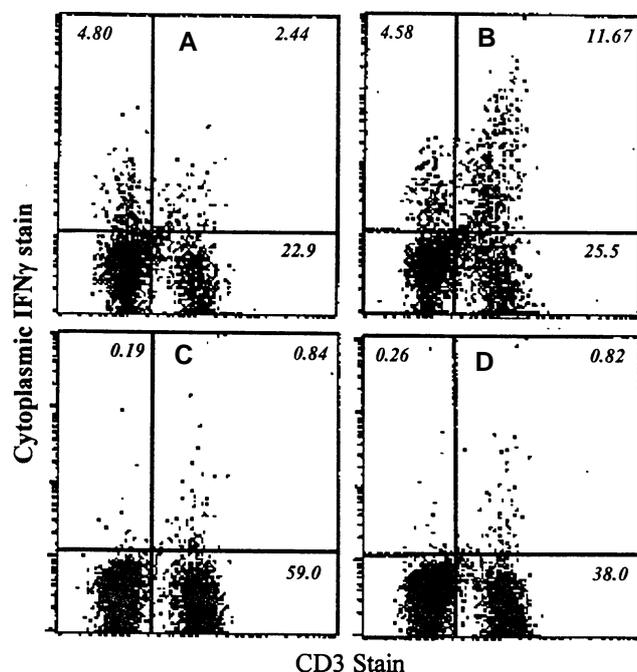


Figure 6. Flow cytometric assessment of cells with cytoplasmic IFN γ in lungs and tracheal LNs of control and BCG infected mice. Lung cells (A and B) and tracheal LNs (C and D) were derived from mice instilled with PBS (A and C) or BCG (B and D) six weeks before sacrifice. Cells were double stained with anti-CD3-FITC and anti-IFN γ -PE. Values in different quadrants denote the percentage of cells in those quadrants.

cytoplasmic staining for IFN γ and (ii) membrane staining with anti-CD3 and cytoplasmic staining for IFN γ . Results of 5 such experiments summarized in table 2 show that the percentages of NK cells making IFN γ closely matched the percentages of CD3 $^-$ cells making IFN γ . Similarly, percentages of CD3 $^+$ cells making IFN γ and NK1.1 $^-$ cells making IFN γ closely matched. The ratio of CD3 $^+$ IFN γ^+ and CD3 $^-$ IFN γ^+ producing cells was 2.18, which was the same for NK1.1 $^-$ IFN γ^+ and NK1.1 $^+$ IFN γ^+ . These results strongly suggest that both NK and T cells contribute to IFN γ production in the lungs of BCG infected mice and that CD3 $^-$ IFN γ^+ cells represent NK cells producing IFN γ .

3.6 Antibody response in BCG infected mice

At each time point after IT instillation of BCG, sera were collected to study the generation of circulating anti-BCG antibodies. Colour development in the anti-BCG antibody ELISA was determined using sera diluted to 1 : 10, 1 : 30 and 1 : 90 (figure 7). Results clearly indicate a lack of substantial antibody response extending until 8 weeks after infection. A significant increase in serum anti-BCG antibody levels were however noted in sera obtained 12 weeks after the initiation of the BCG infection ($P < 0.05$).

4. Discussion

Cellular basis of lung immunity against BCG infection in mouse lungs is beginning to be understood (Wakeham *et al* 1998; Xing *et al* 1998; Fulton *et al* 2000). An increase in T cells in lung interstitium and elevated Th1 cytokines like IFN γ , TNF α and IL12 in broncho-alveolar lavage have been demonstrated (Wakeham *et al* 1998).

Table 2. Comparison of IFN γ producing lymphocytes in BCG infected mouse lungs: CD3 $^+$ IFN γ^+ cells with NK1.1 $^-$ IFN γ^+ and NK1.1 $^+$ IFN γ^+ cells with CD3 $^-$ IFN γ^+ cells*.

Lung cell preparation	Lung derived lymphocytes with described phenotype (%)			
	Stain NK1.1 vs IFN γ		Stain CD3 vs IFN γ	
	NK1.1 $^+$ IFN γ^+	NK1.1 $^-$ IFN γ^+	CD3 $^+$ IFN γ^+	CD3 $^-$ IFN γ^+
1	2.40	5.90	5.30	2.23
2	2.15	7.08	6.12	2.90
3	2.73	6.01	5.25	2.70
4	2.36	5.25	4.58	2.99
5	1.87	4.52	3.78	2.38
Mean \pm SD	2.30 \pm 0.32	5.01 \pm 0.88	5.75 \pm 0.95	2.64 \pm 0.33

*Five lung derived leukocyte preparations derived from mice intratracheally infected with BCG for 5 weeks, were double stained with (i) NK1.1 and IFN γ antibodies, and (ii) CD3 and IFN γ antibodies. NK1.1 and CD3 antibodies were used for surface staining and IFN γ antibody for cytoplasmic stain after fixing/permeabilization of cells. Percent NK1.1 $^+$ and NK1.1 $^-$ cells making IFN γ and percent CD3 $^+$ cells and CD3 $^-$ cells making IFN γ for each cell preparation was estimated by flow cytometric analysis as described in §2.

While IFN γ plays a key role in inducing anti-mycobacterial immunity (Cooper *et al* 1993; Flynn *et al* 1993), the identity of cells which secrete IFN γ , their relative proportions, and the kinetics of their accumulation in lungs have not been established. In the present study, our primary aim was to delineate the kinetics of accumulation of different lymphocyte subsets in the lungs of BCG infected mice and to identify cells containing IFN γ in their cytoplasm, as these cells are expected to secrete the cytokine.

The pattern of changes observed in bacterial load in lungs and other tissues following IT installation of a small number of BCG (2.5×10^4 /mouse) was similar to that reported by others (Wakeham *et al* 1998; Fulton *et al* 2000). Peak bacterial load in lungs was attained between 4 to 6 weeks and it declined to very low levels by the 12th week of infection. These results would suggest that protective immune responses in lungs become effective from the 4th week onwards. The bacterial load in the spleen, which must have been acquired after dissemination of infection via the circulation, increased until the 6th week and persisted at high levels on the 12th week after infection. Sustained infection in the spleen, after the infection has been cleared from the lungs suggests that either the systemic protective responses may take more time to develop than local protective responses after IT instillation of BCG, or that the spleen is a favoured site for growth of BCG.

Timing of initiation of protective immunity in lungs is also suggested by the kinetics of leukocyte influx into the lungs. By the 5th and 6th week after infection, recovery of leukocytes from BCG-infected lungs was double as

compared to control lungs. Interestingly, in spite of doubling of total leukocyte recovery from infected lungs, the relative proportions of granulocytes, lymphocytes and macrophages essentially remained unchanged. A marginal increase in granulocyte proportion on day 1 and of macrophages later on day 2, could reflect the sequence of events observed in most inflammatory reactions (Larsen and Henson 1983). Almost 70% of the leukocytes were lymphocytes. Since lymphocytes are involved in imparting adaptive immunity, we made a detailed analysis of lymphocyte subpopulations present in lungs of control and BCG infected mice. Our results indicate that the lung interstitium accumulates relatively high levels of NK cells (range 14–28% of lung lymphocytes), as compared to some lymphoid tissues like spleen (NK cells < 5%), (Reichlin *et al* 1999). These results corroborate the previously reported higher levels of NK activity in mouse lungs (Wiltout *et al* 1985). Our results also show that a subset of NK cells from control lungs appear to make IFN γ (figure 6), which could either be spontaneous or a consequence of exposure to environmental antigens. It is possible that the purpose of high accumulation of NK cells in lung interstitium is to secrete and maintain a certain basal level of IFN γ in order to afford a protection from viral and other infectious agents commonly encountered in lungs. NK cells are known to play an important role in natural immunity (Trinchieri 1995), and our results support a similar role for NK cells in lungs.

Proportions of T cells increased in BCG infected mice, on expense of NK cells whose proportions declined. In terms of absolute numbers however, both T as well NK cells increased in the lungs of BCG infected mice;

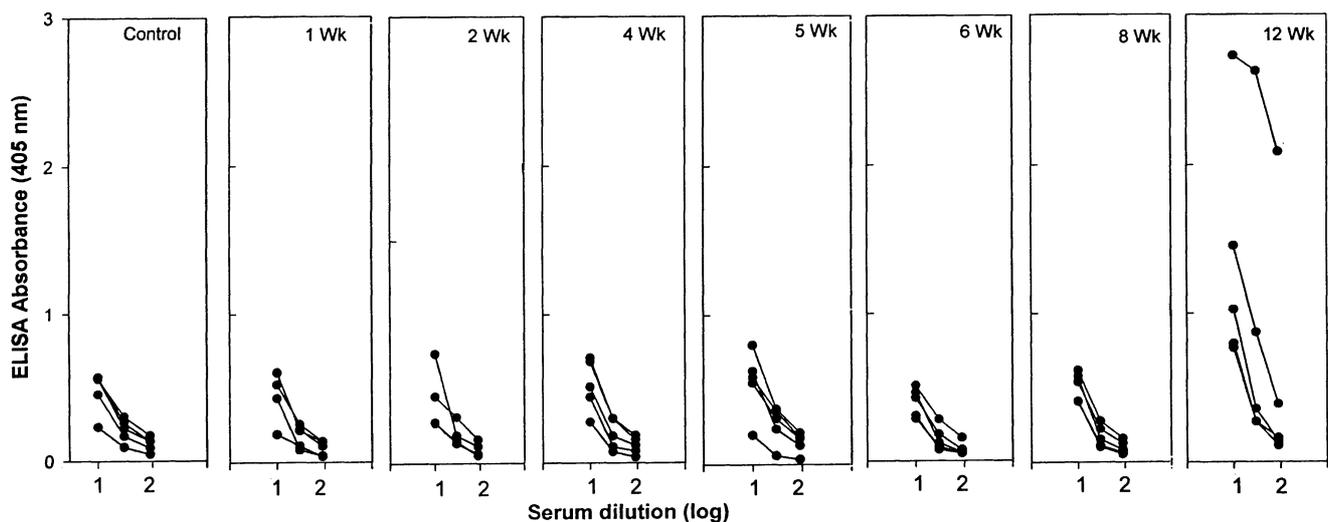


Figure 7. Serum anti-BCG antibody levels after murine pulmonary BCG infection. Anti-BCG antibody levels were determined by ELISA and are expressed as ELISA absorbance at 405 nm. Each panel represents a different time point after infection, and different curves in each panel represent sera from individual animals.

increase in T cells being much higher than that in NK cells. Since greater proportions of T cells make IFN γ in lungs of BCG infected mice, relative enrichment of T cells may be a beneficial response aimed at augmenting the secretion of protective IFN γ locally at the site of infection. Significant numbers of non-T lymphocytes made IFN γ in control as well as BCG infected lungs. Since non-T cells making IFN γ were essentially NK cells, these results indicate that besides T cells, NK cells may also contribute significantly to the overall IFN γ response in lungs of control and BCG infected mice.

A marked increase in the size of lung associated lymph nodes (tracheal LN) was observed in BCG infected mice. Interestingly while the numbers of both T and B cells increased in infected tracheal LNs, the extent of increase was greater for B cells. Mean T/B ratio in tracheal LNs fell progressively from 1.12 in control mice to about 0.61, 12 weeks after BCG infection. It is possible that the relative lower proportions of T cells in tracheal LNs of infected mice was due to migration of activated T cells to lungs and other mucosal sites or to a greater inherent proliferative response of B cells, or both. Interestingly, in contrast to lung cells, few IFN γ secreting cells (< 0.5%) could be demonstrated in tracheal LNs otherwise undergoing massive expansion in response to BCG challenge. Lack of IFN γ production in T cells derived from tracheal LNs and an increased IFN γ secretory response in T cells derived from lungs suggests the occurrence of maturational changes associated with migration to, or residence in the lungs. In this regard, it is well known that intrapulmonary T cells are mostly mature (memory) T cells, while a large proportion of circulating T cells are naive (Agostini *et al* 1993). Cytokine networking with a number of pulmonary cell types, including endothelial cells, fibroblasts, epithelial cells, and macrophages, might account for the development of IFN γ positivity in mature pulmonary effector T cells.

Significant anti-BCG antibody titers were detected in the sera of BCG infected mice 12 weeks after the initiation of infection. These findings are consistent with serologic responses in human TB, where serum anti-TB antibodies do not always increase during latent TB infection but are frequently increased during active disease (Udaykumar *et al* 1991). As the clearance of BCG infection from the lungs occurred prior to the development of a substantial antibody response, our results do not suggest an important role for humoral immune responses in pulmonary host defense against this infection.

Acknowledgment

RKS was supported by a senior associateship award from National research council (NRC), USA.

References

- Agostini C, Chilosi M, Zambello R, Trentin L and Semenzato G 1993 Pulmonary immune cells in health and disease: lymphocytes; *Eur. Respir. J.* **6** 1378–1401
- Andersen P 1997 Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*; *Scand. J. Immunol.* **45** 1009–1031
- Bleed D, Dye C and Raviglione M C 2000 Dynamics and control of the global tuberculosis epidemic; *Curr. Opin. Pulm. Med.* **6** 174–179
- Cooper A M, Dalton D K, Stewart T A, Griffin J P, Russell D G and Orme I M 1993 Disseminated tuberculosis in interferon-gamma gene-disrupted mice; *J. Exp. Med.* **178** 2243–2247
- Cooper A M, Magram J, Ferrante J and Orme I M 1997 IL-12 is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*; *J. Exp. Med.* **186** 39–45
- Fine P E M 1989 The BCG story: lessons from the past and implications for the future; *Rev. Infect. Dis.* **12** 353–359
- Flynn J L, Chan J, Triebold K J, Dalton D K, Stewart T A and Bloom B R 1993 An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection; *J. Exp. Med.* **178** 2249–2254
- Fulton S A, Martin T D, Redline R W and Boom W H 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
- Huygen K, Abramowicz D, Vandenbussche P, Jacobs F, De Bruyn J, Kentos A, Drowart A, Van Vooren J P and Goldman M 1992 Spleen cell cytokine secretion in *Mycobacterium bovis* BCG-infected mice; *Infect. Immun.* **60** 2880–2886
- Ibsen M W, Bakken V, Jonsson R and Hordnes K 1997 Immune responses in mice after gastric and subcutaneous immunization with BCG; *Scand. J. Immunol.* **46** 274–280
- Keane-Myers A, Wysocka M, Trinchieri G and Wills-Karp M 1998 Resistance to antigen-induced airway hyperresponsiveness requires endogenous production of IL-12; *J. Immunol.* **161** 919–926
- Larsen G L and Henson P M 1983 Mediators of inflammation; *Annu. Rev. Immunol.* **1** 335–359
- North R J 1995 *Mycobacterium tuberculosis* is strikingly more virulent for mice when given via the respiratory than via the intravenous route; *J. Infect. Dis.* **172** 1550–1553
- North R J and Izzo A A 1993 Granuloma formation in severe combined immunodeficient (SCID) mice in response to progressive BCG infection. Tendency not to form granulomas in the lung is associated with faster bacterial growth in this organ; *Am. J. Pathol.* **142** 1959–1966
- Orme I M 1999 Beyond BCG: the potential for a more effective TB vaccine; *Mol. Med. Today* **5** 487–492
- Reichlin A, Lizuka K and Yokoyama W M 1999 Isolation of murine natural killer cells; in *Current protocols in immunology* (eds) J E Coligan, A M Kruisbeek, D H Margulies, E M Shevach and W Strober (New York: John Wiley) pp 3-22-1–3-22-6
- Trinchieri G 1995 Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis; *Semin. Immunol.* **7** 83–88
- Udaykumar, Sarin R and Saxena R K 1991 Analysis of circulating immune complexes (CIC) in tuberculosis: Levels of specific antibodies and antigens in CIC and relationship with serum antibody; *FEMS Microbiol. Immunol.* **76** 135–142

- Wakeham J, Wang J, Magram J, Croitoru K, Harkness R, Dunn P, Zganiacz A and Xing Z 1998 Lack of both types 1 and 2 cytokines, tissue inflammatory responses, and immune protection during pulmonary infection by *Mycobacterium bovis* bacille Calmette-Guerin in IL-12-deficient mice; *J. Immunol.* **160** 6101–6111
- Wiltout R H, Herberman R B, Zhang S R, Chirigos M A, Ortaldo J R, Green K M and Talmadge J E 1985 Role of organ-associated N K cells in decreased formation of experimental metastases in lung and liver; *J. Immunol.* **134** 4267–4275
- Xing Z, Wang J, Croitoru K and Wakeham J 1998 Protection by CD4 or CD8 T cells against pulmonary *Mycobacterium bovis* bacillus Calmette-Guerin infection; *Infect. Immun.* **66** 5537–5542

MS received 26 September 2001; accepted 9 January 2002

Corresponding editor: VIDYANAND NANJUNDIAH