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Kinetics of changes in lymphocyte sub-populations in mouse lungs after intrapulmonary infection with *M. bovis* (Bacillus Calmette-Guerin) and identity of cells responsible for IFNγ responses

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SUMMARY

Gamma interferon (IFNγ) plays a key role in host defense against pulmonary mycobacterial infections. A variety of lymphocyte subsets may participate in producing pulmonary IFNγ responses, but their relative contributions after mycobacterial infection have not been clearly elucidated. To address this question, C57Bl/6 female mice were infected by intrapulmonary instillation of $2{\cdot}5 \times 10^4$ BCG (*Mycobacterium bovis* Bacillus Calmette-Guerin). Lymphocyte populations in lung interstitium were examined at different time points after the infection. BCG load in lungs peaked between 4 and 6 weeks post-infection and declined to very low levels by the 12th week of infection. Recovery of lung interstitial lymphocytes doubled by 4–6 weeks after infection and declined thereafter. Flow cytometric analysis of the lung-derived lymphocytes revealed that about 5% of the these cells made IFNγ in control mice, and this baseline IFNγ production involved T ($CD3^+NK1.1^-$), NK ($CD3^-NK1.1^+$) and NKT ($CD3^+NK1.1^+$) cells. As the BCG lung infection peaked, the total number of $CD3^+$ T cells in the lungs increased three-fold at 5–6 weeks post-infection. There was a marked increase (sixfold) in the number of T cells secreting IFNγ 5–6 weeks post-infection. Some increase was also noted in the NKT cells making IFNγ, but the numbers of NK cells making IFNγ in BCG-infected lungs remained unaltered. Our results suggest that whereas NK and NKT cells contribute to baseline IFNγ secretion in control lungs, expansion in the IFNγ-producing T-cell population was essentially responsible for the augmented response seen in lungs of BCG-infected mice.

Keywords Interferon-gamma T cells BCG infection mice host resistance models

INTRODUCTION

Tuberculosis (TB) is a contagious disease initiated by inhalation of airborne droplets with live *Mycobacterium tuberculosis* coughed out by patients with active TB infection. In most infected persons, localized immune responses can clear the bacteria or encapsulate it in granulomas, and active disease is prevented [1,2]. Some live *M. tuberculosis* may, however, survive inside granulomas, and can cause subsequent reactivation of disease, particularly if the immune system is suppressed. Almost one third of all humans are estimated to be infected with *M. tuberculosis*. Three million people die of TB and 8 million fresh cases are detected each year, making it one of the most serious global health problems [3]. The only vaccine available for TB is BCG (*M. bovis*, Bacillus Calmette-Guerin). Its efficacy has been variable, ranging from 0% to 80% in different studies [4,5]. Development of an effective vaccine against TB is hampered by the lack of clear understanding about the mechanism of protective immunity.

A substantial body of literature exists documenting immune responses to systemic challenge with mycobacteria in murine models [6–8]. However, it has been demonstrated that immune responses to systemic and lung infections with mycobacteria are substantially different. For example, mice are almost 100-fold more susceptible to infection initiated in lungs than infection initiated systemically [9]. Since lungs are the portal of entry and the most common site of clinically-apparent TB, it is important to understand the nature of immune responses to mycobacterial infection in lungs. The mouse model of BCG infection in lungs resembles human infection in as much as the infection is effectively contained. Protective immune responses in this model are beginning to be elucidated [10–13]. It is known that infection peaks at 4–6 weeks after intratracheal (IT) deposition of BCG, and there is an associated surge in T cells in the lung interstitium [10,11]. Broncho-alveolar lavage (BAL) contains increased levels of cytokines associated with T-helper (Th)1-type responses, such as IFNγ, IL12 and TNFα [11]. Crucial roles of type I cytokines,

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especially IFNγ in anti-mycobacterial immunity, have been demonstrated using immunodeficient knockout and transgenic mouse models [11,13–19]. Many types of immune cells, like T cells, NK cells and macrophages, can produce Th1 cytokines or cytokines supportive of Th1 responses. The kinetics of activation and accumulation of these cells in lungs of infected mice is not clearly understood. Moreover the relative contribution of different lymphocyte subsets to secretion of Th1 cytokines is unknown.

Our specific aims, therefore, were to document the kinetics of changes in lymphocyte subpopulations after murine pulmonary BCG infection, and to identify cell populations responsible for IFNγ responses. Our results suggest that significant numbers of T, NK and NKT cells in lung interstitium of uninfected control mice make IFNγ. In mice with BCG lung infection, however, augmented IFNγ production in response to BCG occurs primarily in the T-cell population.

MATERIALS AND METHODS

Animals

C57Bl/6 female mice 12–15 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME, 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.

Reagents

Percoll, phorbol 12-myristate 13-acetate (PMA), ionomycin, collagenase (cat no. C5138), DNAse (cat. no. D4263), hyluronidase (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 (MoAbs) labelled with FITC, PE or PerCP [anti-CD3 (Clone 17A2); anti-NK1.1 (clone PK136); anti-CD16/32 (Fc-block, clone 2.4G2); anti-mouse IFNγ (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.

BCG and interpulmonary instillation

A seed culture of BCG (*M. bovis* Pasteur, TMCC no. 1011) was kindly provided by Prof. Ian Orm of the Microbiology Department, Colorado State University, Fort Collins, CO. BCG was grown in Middlebrook culture medium with OADC supplement and Tween. Viable BCG suspensions were counted by plating bacterial suspensions at different dilutions on Middlebrook agar plates and counting colonies after 2 weeks. Mice were anaesthetized by i.p. administration of 0·15 ml of a solution containing Ketamine HCl (8·3 mg/ml) and Xylazine (0·33 mg/ml), and BCG suspension (50 μl bacterial suspension containing $2{\cdot}5 \times 10^4$ bacteria/mouse) was instilled into the lungs of C57Bl/6 mice by the method described by Keane-Myers *et al*. [20]. An equal volume of phosphate-buffered saline (PBS) was similarly administered into control C57Bl/6 mice.

Processing of tissues

Mice were sacrificed by pentobarbital overdose. In order to minimize contamination of lung interstitial leucocyte 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 by Wakeham *et al*. [11] with minor modifications. 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 pressed through a stainless steel mesh. A small aliquot of the suspension for each lung preparation was taken for BCG count and the rest used for isolation of lymphocytes by Percoll density gradient centrifugation [11].

Flow cytometry

Cells derived from lungs were stained with MoAbs against several membrane markers as well as against IFNγ to detect cytoplasmic IFNγ. Cells were distributed at $0{\cdot}2–0{\cdot}3 \times 10^6$ per well of a deep 96-well plate. Cells were washed twice with staining buffer (1% FCS and 0·1% sodium azide in PBS). They were then suspended in 20 μl staining buffer containing 1 μg anti-CD16/32 MoAb (Fc-Block) and incubated at room temperature for 20 min. Staining MoAbs (1 μg in 20 μl staining buffer) were then added and incubation continued for an additional 30 min at room temperature in the dark. Cells were washed twice with staining buffer and fixed by adding 0·1 ml 0·4% paraformaldehyde solution to the loosened pellets of stained cells. For cytoplasmic staining for IFNγ, $0{\cdot}3–0{\cdot}5 \times 10^6$ cells in 200 μl CM were cultured for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin. Fixing/permeabalization and staining of cells for cytoplasmic IFNγ and membrane markers for T or NK cells was done using a kit and the protocol recommended by the manufacturer (Pharmingen). Cells were analysed on a Becton Dickinson FACSCalibur 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.

Statistical analysis

Two-way comparisons were performed using Student's *t*-test. Multiple comparisons were performed by analysis of variance. Comparisons were made using computer software (Sigmastat, SPSS, Chicago, IL, USA). Comparisons were considered significantly different at a level of $P < 0{\cdot}05$.

RESULTS

Recovery of lung interstitial lymphocytes from BCG-infected C57Bl/6 mice

BCG suspension or an equal volume of PBS was instilled in lungs of C57Bl/6 mice. As described in previous studies [10,11], BCG load in the lungs increased and peaked between 4 and 6 weeks post-infection and fell thereafter (results not shown). By the 12th week after infection, BCG were essentially cleared from lungs. Average lymphocyte recovery from control mice ranged between 2 and 3×10^6 cells/lung. At each time point, the recovery of lung interstitial lymphocytes in an infected group of mice was compared with the corresponding recoveries from control PBS-instilled mice. Results in Table 1 indicate that leucocyte recovery from lungs of infected mice was significantly greater than the control recoveries at 5, 6 and 8 weeks after infection. Maximum increase of about 130% was seen at the 5th week post-infection (Table 1).

Table 1. Recovery of lung cells from control and BCG-infected mice

Duration of infection (weeks)	Lung cells recovery from PBS-instilled mice ($\times 10^6$/lung) + s.d.	Lung cell recovery from BCG-instilled mice ($\times 10^6$/lung) + s.d.	Percent increase
2	2·30 + 0·63	3·06 + 0·58	33·0
4	2·36 + 0·41	3·17 + 0·36	34·3
5	2·50 + 0·43	5·79 + 0·87	131·6*
6	2·34 + 0·76	4·80 + 0·86	93·5*
8	2·94 + 0·88	4·76 + 0·83	61·9*
12	2·62 + 0·60	3·72 + 0·83	42·0

BCG ($2{\cdot}5 \times 10^4$/mouse) or PBS was instilled in lungs of C57Bl6 mice. At each time point after BCG/PBS instillation, lung lymphocytes were isolated from 5–9 control and BCG-infected mice.

*$P < 0{\cdot}05$.

Lymphocyte sub-populations in the lungs of BCG-infected mice

Flow cytometric analysis was used to enumerate lymphocyte sub-populations in lungs at each time point. For all phenotypic studies, a lymphocyte gate was set on the forward/side scatter plot (Fig. 1a). Cells were stained and analysed for a variety of phenotypic markers. Results in Fig. 1 show a set of typical flow cytometry results of cells double-stained for CD3 and NK1.1 markers (Fig. 1b), CD4 and CD8 markers (Fig. 1c), and CD3 and cytoplasmic IFNγ (Fig. 1d). Significant variations were found in the proportions of various lymphocyte sub-populations derived from lungs. Data in Table 2 show the average representation of T, NK, NKT, B and $\gamma\delta$T cells in lung interstitial cell preparations derived from control mice. The average CD4/CD8 cell ratio and its range is also given in Table 2.

Knowing the values of total cell recovery from lungs and percentage of a particular cell sub-population by flow cytometry, absolute numbers of lymphocyte sub-populations per lung could be computed. Absolute numbers of T cells ($CD3^+$), NK cells ($NK1.1^+$), T cells with cytoplasmic IFNγ ($CD3^+IFN\gamma^+$) and non-T cells with cytoplasmic IFNγ ($CD3^-IFN\gamma^+$) in lungs of control and BCG-infected mice at different time points after infection are shown in Fig. 2. These results indicate that absolute numbers of $CD3^+$ and $NK1.1^+$ cells per lung increased significantly in BCG-infected mice, specifically 5 and 6 weeks post infection (Fig. 2). A marked increase in the $CD3^+IFN\gamma^+$ population occurred in BCG-infected mice (Fig. 2c), maximum increase being about sixfold 5–6 weeks post-infection. The extent of increase in $CD3^+IFN\gamma^+$ cells was greater than the increase in total $CD3^+$ cells (about threefold) in BCG-infected mice. Interestingly, a significant number of $CD3^-$ cells derived from control or BCG-infected mice were positive for cytoplasmic IFNγ (Fig. 2d). These results suggest that non-T cells (e.g. NK cells) may contribute to IFNγ production in control and BCG-infected mouse lungs. No significant change was observed in the relative proportions of $\gamma\delta$T cells and B cells, and in CD4/CD8 ratios in lymphocyte preparations derived from control or BCG-infected mouse lungs (results not shown).

While $CD3^+$ cells secreting IFNγ increased considerably in BCG-infected lungs (Fig. 2), these could include NKT ($CD3^+NK1.1^+$) cells, whose contribution, if any, to IFNγ secretion could not be assessed by these results. Three-colour flow cytometric analysis was carried out to further characterize the cells which make IFNγ, and to assess the relative contributions of T and non-T cells to IFNγ production in lung cells derived from control and BCG-infected (5 week post-infection) mice. For this purpose, lung cells were stained with anti-CD3 (perCP), anti NK1.1 (FITC) and anti-IFNγ (PE) antibodies. From the CD3 *versus* NK 1.1 plots, gates were put on NK cells ($CD3^-NK1.1^+$), T cells ($CD3^+NK1.1^-$) and NKT ($CD3^+NK1.1^+$) cells, and these three populations were further analysed for cytoplasmic staining for IFNγ. Results of these experiments summarized in Table 3 indicate that on average, absolute numbers of T cells ($CD3^+NK1.1^-$) as well as T cells making IFNγ ($CD3^+NK1.1^-IFN\gamma^+$ population) increased by three- and sevenfold, respectively. While absolute numbers of NK cells ($NK1.1^+CD3^-$) per lung increased in BCG-infected mice, numbers of IFNγ-producing NK cells ($CD3^-NK1.1^+IFN\gamma^+$ population) remained unaltered (Table 3). NKT cells ($NK1.1^+CD3^+$) constituted a small population in control as well as BCG-infected lungs, yet absolute numbers of IFNγ-producing NKT cells ($CD3^+NK1.1^+IFN\gamma^+$ population) appear to increase in BCG-infected lungs (Table 3).

Table 2. Representation of various lymphocyte sub-populations in lung interstitial cell preparations

Cell population	Average representation* (%) Mean + s.d.	Range
T cells ($CD3^+NK1.1^-$)	24·3 + 2·90 (34)†	18·1–31·1
NK cells ($CD3^-NK1.1^+$)	18·8 + 3·84 (28)	8·9–25·1
NKT cells ($CD3^+NK1.1^+$)	2·2 + 0·73 (18)	1·0–3·5
B cells (sIg^+)	42·7 + 2·36 (5)	42·7–49·0
$\gamma\delta$ T cells	1·89 + 0·43 (15)	1·2–2·6
CD4/CD8 ratio	1·20 + 0·21 (20)	1·0–1·9

*Cell preparations derived from lungs were stained for various given markers and analysed on a flow cytometer.

†Number of cell preparations analysed.

DISCUSSION

Most people exposed to *M. tuberculosis* can effectively fight the infection, and it is only a small fraction of infected persons that develops active disease. The mouse model of BCG lung infection

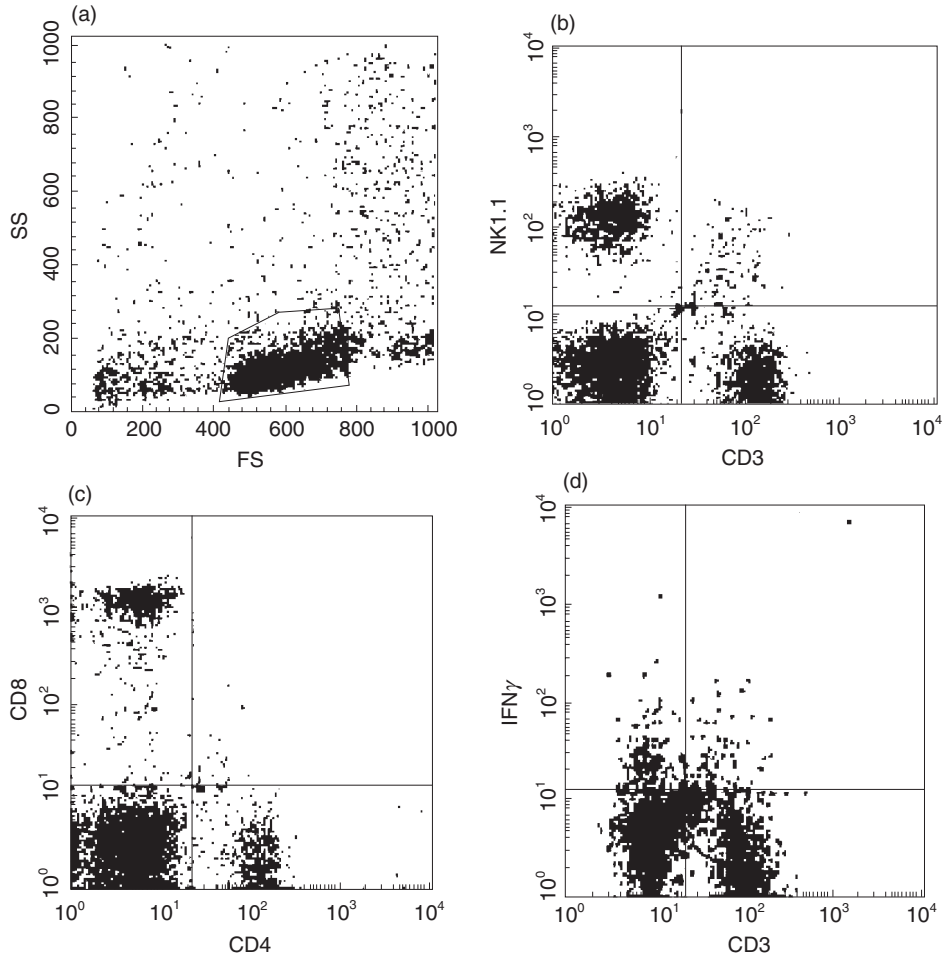

Fig. 1. Representative flow cytometric results showing discrimination of various cell sub-populations in interstitial cell preparations derived from normal mouse lungs. The lymphocyte gate used is shown in (a). Lung cells were stained with different pairs of phenotyping antibodies, CD3 and NK1.1 markers (b), CD4 and CD8 markers (c), CD3 and cytoplasmic IFNγ (d). Antibodies were tagged with FITC (*x* axis) and PE (*y* axis).

Table 3. Effect of BCG lung infection on IFNγ production by different lymphocyte sub-populations in lung interstitium of C57Bl/6 mice

Treat-ment	Total lung cell recovery ($\times 10^6$)	Cell sub-population				Percent cells with cytoplasmic IFNγ	IFNγ-producing cells per lung ($\times 10^5$)
		Sub-population	Phenotype	Percentage of lung cells	Cells/lung ($\times 10^5$)		
PBS	2·52 + 0·41	T cells	$CD3^+NK1{\cdot}1^-$	25·44 + 3·71	6·53 + 1·84	17·03 + 4·66	1·06 + 0·15
		NK cells	$CD3^-NK1{\cdot}1^+$	12·88 + 4·04	3·13 + 0·58	33·06 + 5·58	1·04 + 0·25
		NKT cells	$CD3^+NK1{\cdot}1^+$	1·30 + 0·20	0·33 + 0·05	50·65 + 16·54	0·16 + 0·05
BCG	6·57 + 0·68	T cells	$CD3^+NK1{\cdot}1^-$	33·00 + 0·87	21·68 + 1·93	33·14 + 5·58	7·01 + 1·64*
		NK cells	$CD3^-NK1{\cdot}1^+$	9·48 + 1·78	6·32 + 1·78	15·94 + 4·46	0·95 + 0·08
		NKT cells	$CD3^+NK1{\cdot}1^+$	2·14 + 0·45	1·40 + 0·27	32·23 + 10·45	0·50 + 0·10*

Lung cells were prepared from control (PBS-instilled) or BCG-infected mice (5 week post-infection) as described in Materials and Methods. Cells were membrane-stained with anti-CD3-PerCP and anti-NK1.1-FITC antibodies followed by intercellular staining with anti-IFN-PE antibody. Relative proportions of NK, T and NKT populations were obtained from two-colour dot matrix plot (FITC *versus* PerCP). These populations were further gated and analysed individually for staining with intercellular IFN (third colour, PE). Relative proportions of different cell populations and their absolute numbers in lungs were calculated. All values are mean + s.d. of four independent experiments. *$P < 0{\cdot}05$ (difference between control and BCG groups).

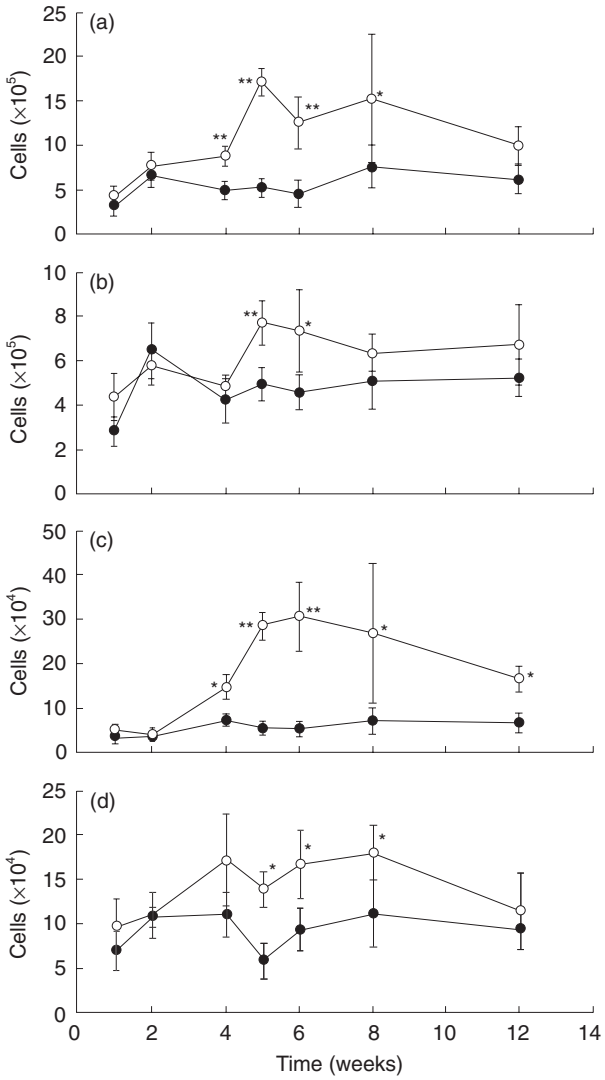

Fig. 2. Kinetics of changes in the absolute recoveries of total $CD3^+$ and $NK1.1^+$ lymphocytes as well as $CD3^+IFN\gamma^+$ and $CD3^-IFN\gamma^+$ lymphocytes from the lungs of control and BCG-infected mice. In each panel, closed circles denote the cell recoveries per lung from control mice and open circles represent the corresponding recoveries from mice infected with BCG. Each value of cell recovery is a mean + s.d. of five observations. BCG group difference from the control group by applying unpaired *t*-test was $P < 0{\cdot}05$* or $P < 0{\cdot}001$**. (a) $CD3^+$ cells; (b) $NK1.1^+$ cells; (c) $CD3^+IFN\gamma$ cells; (d) $CD3^-IFN\gamma$ cells.

resembles human TB in that the infection is self-limiting. Some information is available in the literature about the cellular basis of lung immunity against BCG infection in mouse lungs [10–13]. An increase in T cells in lung interstitium, and elevated Th1 cytokines like IFNγ, TNFα and IL12 in BAL, have been demonstrated [11]. IFNγ appears to have a crucial role in innate as well as acquired immunity against mycobacterial immunity [13–18]. T cells and NK cells are two prominent cell types which have the ability to secrete IFNγ, but their relative contribution to the IFNγ response in mycobacterial infection is not clear. IL-15 transgenic mice were recently shown to have elevated levels of NK cells as well as IFNγ levels, and were relatively resistant to mycobacterial infection [19]. Interestingly, depletion of NK cells as well as T cells in IL-15 transgenic mice resulted in increased susceptibility to BCG [19], indicating that both cell types may have an important role in anti-mycobacterial immunity. 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 IFNγ in their cytoplasm, as these cells are expected to secrete the cytokine.

As documented in previous reports [10,11], peak bacterial load in lungs was attained between 4 and 6 weeks and 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. Timing of initiation of protective immunity in lungs is also suggested by the kinetics of lymphocyte influx into the lungs. By the 5th and 6th week after infection, recovery of lymphocytes from BCG-infected lungs was double that from control lungs. The relative proportion of NK cells ($NK1.1^+$) in lung interstitium (Table 2,3) is significantly higher than the proportion of these cells in spleen (range 1–3%, results not shown). These results corroborate the previously reported higher levels of NK activity in mouse lungs as compared with spleen [21]. Our results further show that about 5% of the lung interstitial lymphocytes from control mice make IFNγ (Table 3), and NK as well as T cells contribute to IFNγ in equal proportion in control lungs (Table 3). Production of IFNγ in control lungs could be spontaneous, or a consequence of exposure to environmental antigens, or both. It is possible that one of the purposes of high accumulation of NK cells in lung interstitium is to secrete and maintain a certain basal level of IFNγ in order to afford protection from viral and other infectious agents commonly encountered in lungs. NK cells are known to play an important role in natural immunity [22], and our results support a similar role for NK cells in lungs. Additionally, IFNγ secretion could have other homeostatic functions, like sustaining a basal level of nitric oxide synthase activity in airway epithelial cells [23].

After BCG infection, the IFNγ-producing cell population expands and at the peak of infection (5 weeks post-infection), about 13% of lung-derived cells are positive for cytoplasmic IFNγ (Table 2). Since there is a significant increase in total lymphocytes recovered from infected lungs, absolute numbers of IFNγ-producing cells per lung increase about fourfold. Almost all the increase in IFNγ production in BCG-infected lungs appears to be due to expansion of an IFNγ-producing subset of T lymphocytes, which increase about sevenfold, whereas the number of NK cells making IFNγ in BCG-infected lungs is not significantly different from the corresponding numbers in control lungs. Absolute numbers of NKT cells making IFNγ in BCG-infected mice appears to increase even though the relative contribution of NKT cells as a source of IFNγ remains low in BCG-infected mice.

The important role of IFNγ as a component of protective immunity to mycobacterial infections is well established [14,17].We have demonstrated that T, NK and NKT cells in the lung may participate in maintaining a base line IFNγ secretion in control lungs. An adaptive immune response to BCG in terms of IFNγ production is, however, almost entirely due to an expansion of IFNγ-producing T cells.

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