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Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines

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SUMMARY

The pattern of cytokines produced by T cells from mice with latent tuberculosis and during reactivation of tuberculosis was determined. A type 1 cytokine pattern was observed in T cells isolated from the lung of mice with latent disease. Reactivation of mycobacterial growth, by activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulted in a shift from a type 1 to a type 2 cytokine pattern in both CD4 and CD8 T cells. Classification of the T cells based on their differential expression of CD45 and CD44 showed that the phenotypically different populations of CD4 and CD8 cells exhibited a type 1 cytokine pattern at latency and that reactivation of latent tuberculosis was associated with a shift in cytokines produced by these populations to a type 2 cytokine response. Control of mycobacterial growth resulted in a return to the type 1 cytokine pattern found during latent disease.

Keywords tuberculosis type 1 and type 2 cytokines latent tuberculosis reactivation

INTRODUCTION

Infection of man by *Mycobacterium tuberculosis* is often characterized by the development of a latent infection. Thus, the immune response controls the growth of the microorganism, perhaps for the life time of the infected individual [1–14]. It is not known presently whether latency is really characterized by a steady state infection in which equal numbers of bacteria divide as are killed, or whether the microorganisms enter a dormant state [15]. Whatever the case, infected individuals stand a 10% life time chance that the disease will reactivate and clinically active tuberculosis (TB) will occur. The factors that are responsible for reactivation are not known but are probably the result of a temporary suppression of the immune response [11,16–19].

Control of TB is thought to be mediated by interferon-gamma (IFN-γ)-producing T cells [3,4,8–10,20–22]. Knock-out mice that cannot produce IFN-γ or that cannot express the IFN-γ receptor are more susceptible to the growth of *M. tuberculosis* then are intact wild-type control mice [23–27]. Both CD4 and CD8 T lymphocytes are important in controlling the growth of the tubercle bacillus. Generally, CD4 T cells are considered to be cytokine-producing helper T cells while CD8 cells are cytolytic T cells. Both populations, however, can produce cytokines. The production of different cytokines by CD4 cells has led to their classification as either Th1 or Th2 cells [28–30]. Th1 cells produce predominantly IL-2, tumour necrosis factor-beta (TNF-β) and IFN-γ and these are important in the generation of cell-mediated immunity. Th2 cells have been shown to produce IL-4, IL-5, IL-6, IL-10 and other cytokines that promote humoral immune responses. A third population of CD4 cells, Th0 cells, has been shown to produce combinations of both Th1 and Th2 cytokines [28–34].

Previous studies in our laboratory have shown that infection of mice by either the intravenous route or intranasally with low doses of *M. tuberculosis* results in the establishment of a latent or steady-state infection which persists for up to 9 months in infected animals [35,36]. An initial period of mycobacterial growth, which reaches peak levels at 6 weeks after infection, is followed by a decline in the number of bacteria that can be isolated from the lungs or spleen, until a steady state or latent infection is attained by week 12 after infection. A temporary suppression of the immune response by activation of the hypothalamic–pituitary–adrenal (HPA) axis resulted in the re-initiation of growth of the bacillus. Previously we have characterized the phenotypic changes that occur in T cell subpopulations during the progression of disease from latency to active growth followed by the reestablishment of immunological control [36]. Reactivation was correlated with a decrease in both CD4 and CD8 naive, activated and memory cells in the lung. The classification of these cells as naive, activated or memory was based on their differential expression of CD44 and CD45RB [37–39]. We also found that the establishment of latency correlated with the production of type 1 cytokines by both CD4 and CD8 T cell populations [40]. The present study extends our previous observations by characterizing the changes in cytokines present in these populations following reactivation of mycobacterial growth.

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MATERIALS AND METHODS

Mice

Specific pathogen-free male BALB/c mice were obtained from Charles River Labs (Wilmington, MA). Mice were 6–8 weeks of age at the start of each experiment. The animals were housed under sterile conditions in microisolator cages (Lab Products, Maywood, NJ) in a BSL-3 facility and given food and water *ad libitum.*

Mycobacterial infection

Mycobacterium tuberculosis (Erdman) was obtained from the American Type Culture Collection (Rockville, MD; ATCC 35801, TMC 107) and grown as previously described [35,36]. Prior to use the microorganisms were thawed, sonicated at 70 cycles/s at 50% efficiency and diluted in Hanks' balanced salt solution (HBSS). Mice were infected intranasally under anaesthesia (Ketamine/Xylacine mixture; Ketamine 48 μg/g (Vedco, St Joseph, MO), Xylacine 3·2 μg/g (Bayer, Shawnee Mission KS)) in 25 μl intramuscularly. The mice were inoculated with 25 μl of the bacterial suspension containing 1000 colony-forming units (CFU) placed on the external nares using a micropipette as described by Saunders & Cheers [41]. The bacteria are inhaled. The number of microorganisms used was confirmed by plate count. Bacterial growth was monitored by plate count by determining the number of CFU in the lung and spleen at various times after inoculation. The course of this infection in the lungs, leading to the establishment of latency and following reactivation, has been described elsewhere [36]. While the terms latency and reactivation have not been clearly defined, latency is used here to define a steady-state infection in which the numbers of bacteria isolated from the lungs of mice remain fairly constant over an extended period of time. Reactivation is used to describe the increase in mycobacterial growth that occurs after activation of the HPA axis. For the purposes of this study time 0 represents latent disease, with each succeeding time point representing the time following the initial period of activation of the HPA axis.

Activation of the HPA axis

The HPA axis was activated as previously described using a restraint paradigm [17,35,42,43]. Briefly, mice were placed into well ventilated 50-ml conical centrifuge tubes overnight. The mice were restrained for five daily 15-h cycles, rested for 2 days and then restrained again for five 15-h cycles. The mice, which were placed in conventional housing at the end of each daily episode of HPA activation, immediately resumed feeding and grooming. Control mice were also housed without access to food and water.

Preparation of lymphocyte populations

Mice were killed at selected time points following HPA activation and single-cell suspensions were prepared from the spleen, mediastinal and superficial cervical lymph nodes as previously described [36]. The lung tissue was cut into pieces and then digested for 45 min with a prescreened lot of collagenase (150 U/ml; Worthington Biochemical, Cleveland, OH). The lymphocytes were purified from the lung cell suspension by gradient centrifugation [36,40]; cells were layered onto 5 ml of Ficoll–Paque (Pharmacia, Piscataway, NJ) in a 15-ml conical centrifuge tube and centrifuged at room temperature for 20 min at 1500 ***g***. This removed cell debris, dead cells and other connective tissue from the lymphocyte population. CD4 and CD8 T lymphocytes were then purified using the IMMULAN mouse T cell subpopulation kit for CD4/CD8 (Biotecx Labs Inc., Houston, TX). The purity of the enriched lymphocyte subsets was confirmed by flow cytometric analysis and was always > 90%.

Flow cytometric analysis

Flow cytometric analysis was used to determine the expression of CD3, CD4 and CD8 to check the efficacy of the separation for the control of samples [36]. In separate staining procedures fluorochrome-conjugated antibodies to CD44, CD45RB, IL-2, IFN-γ, IL-4 and IL-10 were used to distinguish the cell populations (Pharmingen (San Diego, CA): anti-CD3, cychrome-conjugated; anti-CD8, PE-conjugated; biotinylated anti-CD45RB; anti-CD44, cychrome-conjugated; IL-2 and IFN-γ, FITC-conjugated; IL-4 and

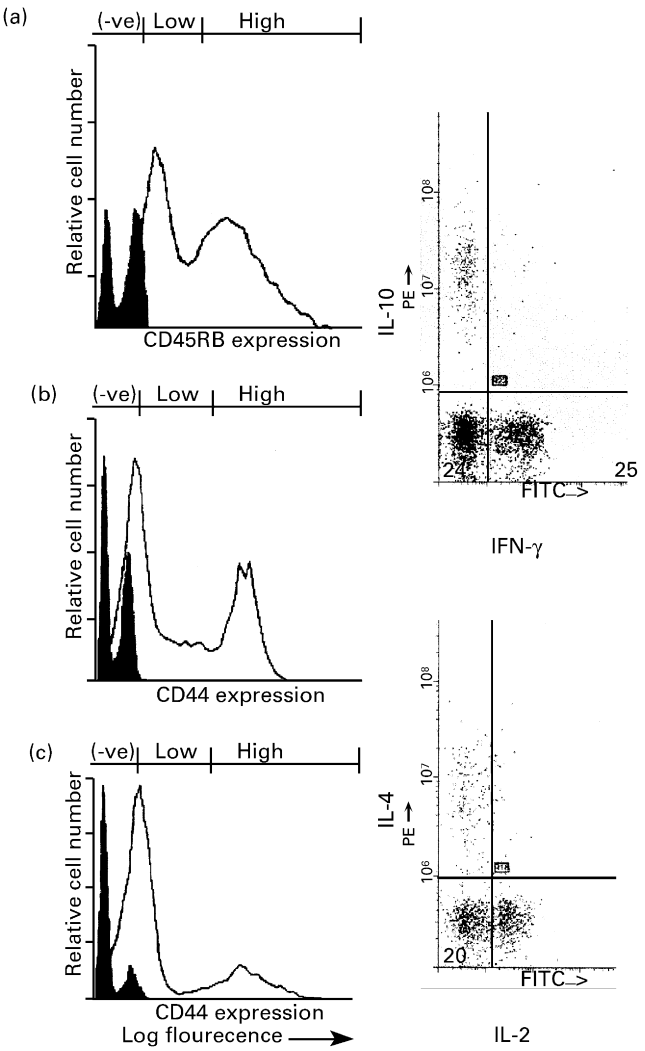

Fig. 1. Flow cytometric analysis of $CD4^+$ T cell subpopulations isolated from the lungs of mice during reactivation of tuberculosis. $CD4^+$ cells were isolated using an IMMULAN mouse T cell isolation kit. Subpopulations of $CD4^+$ cells were identified by the differential expression of CD45 and CD44 and the cytokines identified following permeabilization of the cells and the use of fluorochrome-labelled cytokine-specific antibody. (a) CD45 expression by CD4 T cells. (b) Expression of CD44 by the $CD45^{lo}$ population. (c) Expression of CD44 by the $CD45^{hi}$ population. The scatter plots are an example of intracellular cytokine analysis taken from a population of CD4, $CD45^{lo}/CD44^{lo}$ cells at latency.

IL-10, PE-conjugated; strepavidin conjugated to RED 613 was obtained from GIBCO-BRL (Burlingame, CA)) and used as described previously by Howard & Zwilling [40]. Purified CD4 and CD8 T cells were analysed for the expression of CD45RB and CD44 and then analysed for the presence of intracellular cytokines. Cells from a pool of three mice were analysed separately and the data represent the means of three different time course experiments. Three-colour (controls) and four-colour (test) multiparameter analysis was performed using a Coulter EPICS Elite flow cytometer (Coulter Corp., Hialeah, FL) as described by Howard *et al*. [40]. For efficacy controls, the T cells were identified by gating of the $CD3^+$ cells. Those cells were analysed for CD4 and CD8. In the test samples, purified CD4 and CD8 T cells were further analysed for the expression of CD45RB and CD44. The MoAbs to CD44 and CD45RB revealed a bimodal staining pattern which resulted in cells with bright and dim intensities indicating the presence of distinct phenotypic populations shown in the contour plot in Fig. 1. Each subset was then analysed for the presence of intracellular cytokines. The separation of the cells expressing the intracellular cytokines can be easily distinguished, as also shown in Fig. 1. The data are expressed as the percentage of each population expressing either IL-2 and IFN-γ (type 1 cytokines) or IL-4 and IL-10 (type 2 cytokines) based on the total number of CD4 or CD8 cells [36,40].

Statistical analysis

The differences in the pattern of cytokine production by the T lymphocyte populations at each time point were determined by one way analysis of variance (ANOVA) (StatMost, Salt Lake City, UT). The differences in the cytokine pattern of the T lymphocyte populations from mice with latent infection were evaluated as was the effect of time on changes in these cytokine patterns. The changes in cytokines produced by the T cells from mice with latent disease did not differ as a result of time and these were collapsed and expressed as a single value as time 0 prior to activation of the HPA axis. The changes in cytokines produced by the T cell subpopulations that resulted from activation of the HPA axis were also analysed by ANOVA. The results were significant if they equaled or exceeded the 95% confidence level ($P \leq 0{\cdot}05$).

RESULTS

Cytokine production by T cell populations in the lungs of mice following reactivation of TB

The cytokines produced by CD4 and CD8 lymphocyte populations isolated from the lung are shown in Figs 2 and 3. The response of the CD4 and CD8 populations expressing $CD45^{lo}/CD44^{lo}$ favoured a Th1 response characterized by the production of IL-2 and IFN-γ; the CD4 Th1 cells outnumbered the Th2 cells by a 3–1 margin at

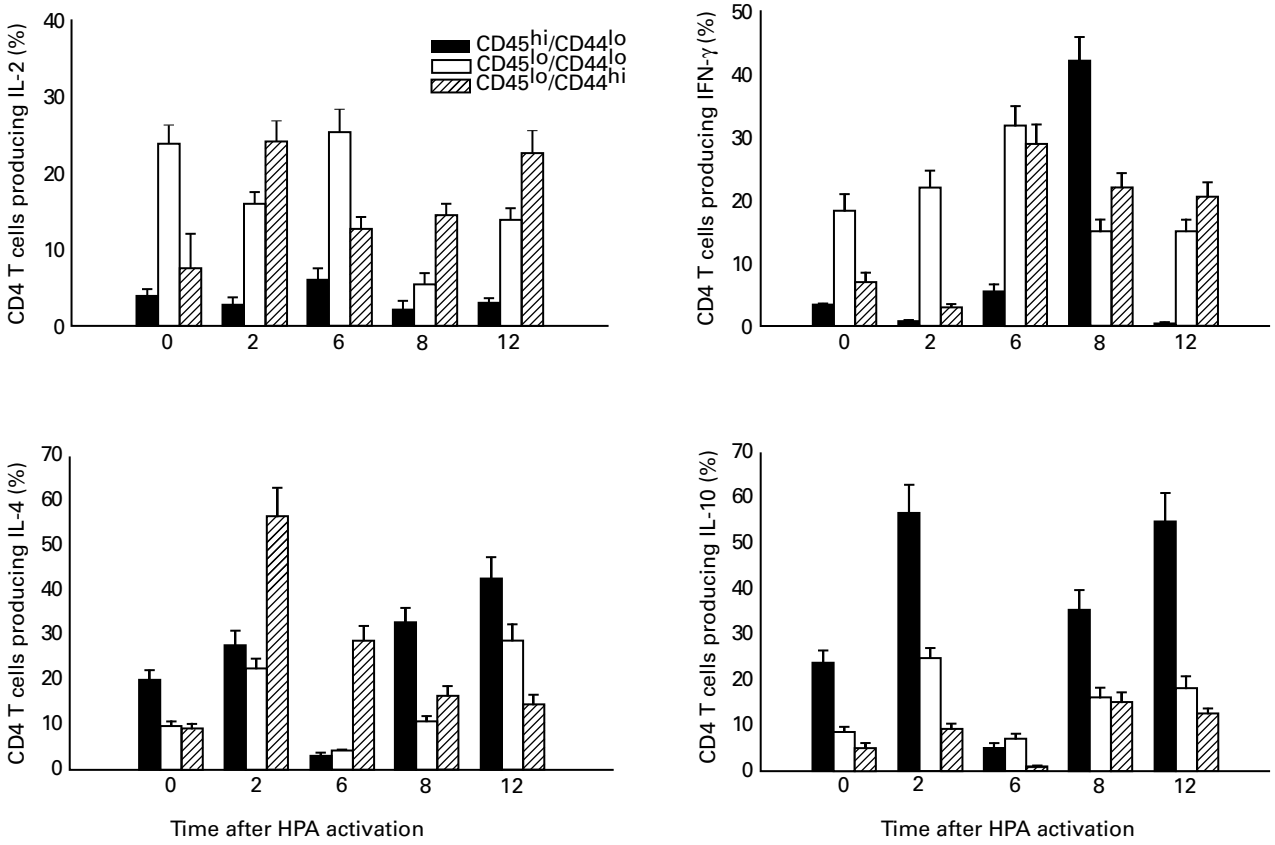

Fig. 2. Cytokine production by $CD4^+$ T cell subpopulations isolated from the lungs of mice during reactivation of tuberculosis. T cells were isolated and characterized as described in the legend for Fig. 1. The effect of hypothalamic–pituitary–adrenal (HPA) activation on the changes in the cytokines produced by the CD4, $CD45^{lo}/CD44^{lo}$ population producing IL-2 and IFN-γ from mice with latent disease was significant ($P < 0{\cdot}001$), as were the changes in the $CD45^{lo}/CD44^{hi}$ population ($P < 0{\cdot}001$). The changes in the Th2 cytokines produced by each of these populations following activation of the HPA axis were also significant ($CD45^{hi}/CD44^{lo}$, $P < 0{\cdot}001$; $CD45^{lo}/CD44^{lo}$, $P < 0{\cdot}04$; $CD45^{lo}/CD44^{hi}$, $P < 0{\cdot}001$).

latency. Immediately following HPA activation at 2 weeks there was a decrease in the percentage of $CD45^{lo}/CD44^{hi}$ cells producing IFN-γ. The percentage of IL-4-producing cells in the $CD45^{lo}/CD44^{hi}$ population increased immediately after activation of the HPA axis, as did the production of IL-10 in the $CD45^{hi}/CD44^{lo}$ population.

The changes in the pattern of cytokines produced by the CD8 population mimicked that observed in the CD4 cells (Fig. 3). A decrease in the percentage of cells containing IL-2 or IFN-γ was observed immediately following activation of the HPA axis at 2 weeks. The percentage of CD8 cells producing the type 2 cytokines IL-4 and IL-10 also increased, especially those containing IL-10. When microbial growth peaked at 6–8 weeks [36], the percentage of cells producing type 1 cytokines began to increase while those producing type 2 cytokines decreased. By week 12, when the numbers of bacteria isolated from the lung returned to levels observed prior to HPA activation [36], the percentage of cells expressing $CD45^{lo}/CD44^{lo}$ and $CD45^{lo}/CD44^{hi}$ producing IL-2 and IFN-γ increased, while those containing IL-4 and IL-10 decreased. Thus, the return to low numbers of mycobacteria was synonymous with a type 1 cytokine response by both CD4 and CD8 T cells.

DISCUSSION

The results of this study indicate that as the numbers of mycobacteria isolated from the lung increase following activation of the HPA axis, there is a change in cytokine production by T cells from predominantly type 1 cytokines to a type 2 cytokine profile. While the cytokines produced by T cells in the lung during latent infection were predominantly type 1, i.e. more IL-2- and IFN-γ-producing cells, HPA activation resulted in a shift to IL-4- and IL-10-producing cells. CD44 and CD45 expression was used to distinguish populations of T cells that have been characterized as naive, activated and memory cells [37–39]. These cells produced distinctly different cytokines and were also affected differently by HPA activation. During latency the $CD4^+$, $CD45^{lo}/CD44^{lo}$ population in the lung produced mostly type 1 cytokines. The temporary suppression of immunity that resulted from HPA activation also resulted in a shift from type 1 activated cells to more cells that produced predominantly type 2 cytokines. This change in the pattern of cytokine production by the activated and memory $CD4^+$ cells was temporary and the cytokine profile shifted back to a type 1 response as bacterial growth peaked and then declined to levels detected at latency [36]. The combination of activated and memory cell populations producing type 1 cytokines was 2–5 times greater then the number of cells producing type 2 cytokines.

The use of CD45 and CD44 to differentiate $CD4^+$ cells into three populations of naive, activated and memory cells revealed that these cells produced different cytokines during latency when analysed *ex vivo*. Thus, the $CD45^{hi}/CD44^{lo}$ population produced predominantly type 2 cytokines while the $CD45^{lo}/CD44^{lo}$ and the $CD45^{lo}/CD44^{hi}$ populations of cells produced predominantly type 1 cytokines. This also proved to be the case for $CD8^+$ T cells. In this case, the different phenotypes differentiated by CD45 and CD44 expression all produced predominantly type 1 cytokines at latency. This observation is similar to that reported by Andersen *et al.* [44] and by Orme and colleagues [8–10,45], who showed that memory cells from *M. tuberculosis*-infected mice, rescued by antibiotic treatment and challenged with tubercle bacilli, produced a type 1 cytokine response. Similarly, the control of mycobacterial growth has been shown to be correlated with the production of the type 1 cytokines IFN-γ and TNF-β [46–49].

The classification of CD8 cells into type 1 and type 2 subtypes has received less attention because these cells are considered to be primarily cytotoxic effector cells. The accelerated growth of *M. tuberculosis* in β_2-microglobulin knock-out mice, that are devoid of CD8 cells, indicates that these cells play an important role in the control of TB in mice [25]. We have already shown that CD8 cells accumulate in the lung of mice infected with *M. tuberculosis* [36]. Our results show that the differential expression of CD45 and CD44 proved useful in distinguishing CD8 cells that produce different cytokine patterns that are analogous to those produced by CD4 T cells. This observation is similar to that reported by Croft *et al.* [39], who showed that CD8 cell cytokine profiles can also be classified into type 1 or type 2 responses. Latency and control of mycobacterial growth were associated with the production of type 1 cytokines by CD8 cells.

It has been difficult to distinguish clearly the cytokine response of T cells from patients infected with TB [3,20,50,51]. Peripheral blood mononuclear cells (PBMC) from healthy, purified protein derivative (PPD)-positive patients, i.e. those with latent disease, also produced a type 1 cytokine response, as did T cells from *M. leprae*-resistant tuberculoid leprosy patients. T cells from patients with lepromatous leprosy, in contrast, produced a predominantly type 2 (IL-4) cytokine response [52]. A more recent study by Torres *et al.* [50] found that patients with active TB produced a predominantly type 2 cytokine response, while PPD^+ household contacts, without active disease, produced a type 1 cytokine response. Studies in mice indicate that early type 1 cytokine responses are followed by the emergence of cells that produce predominantly type 2 cytokines [10,53]. These results and those reported previously [40] show that a type 1 cytokine response predominates when mycobacterial growth is controlled. This is followed by a type 2 cytokine response when the numbers of bacteria are increasing. As control of bacterial growth was re-established, a type 1 cytokine response again predominated.

The shift in T cell cytokine profiles from a Th1 to a Th2 profile following activation of the HPA axis is probably responsible for the reactivation of mycobacterial growth. Activation of the HPA axis results in an increase in corticosteroids which are widely used as immunosuppressive agents [54]. Corticosterone has been shown to induce apoptosis in T cells [55–58], to suppress cytokine production and to effect a shift in cytokine profiles from Th1 to Th2 [59,60]. The production of type 1 cytokines is controlled by the presence of IL-15 produced by natural killer (NK) cells [61–64] as well as the production of IFN-γ by Th1 cells and NK cells and the production of IL-12 by macrophages [65,66]. The decreased cellularity in the lung and lymphoid organs following HPA activation [54,55] is probably an indication that increased glucocorticoids resulted in a depletion of T cells resulting in the re-initiation of the growth of the tubercle bacillus. Corticosterone suppression of Th1-inducing cytokines may have resulted in the initial differentiation of T cells along the Th2 pathway. For example, the change in the cytokine profile could have been the result of a suppression of IL-12 production by macrophages as a result of high glucocorticoid levels [67–69]. The reappearance of Th1 cells, that was associated with peak bacterial loads, may have been the result of the differentiation of activated Th0 cells along the Th1 pathway. The Th0 population that produced both IFN-γ and IL-10 was apparent immediately after HPA activation and could differentiate into either a Th1 or a Th2 pathway

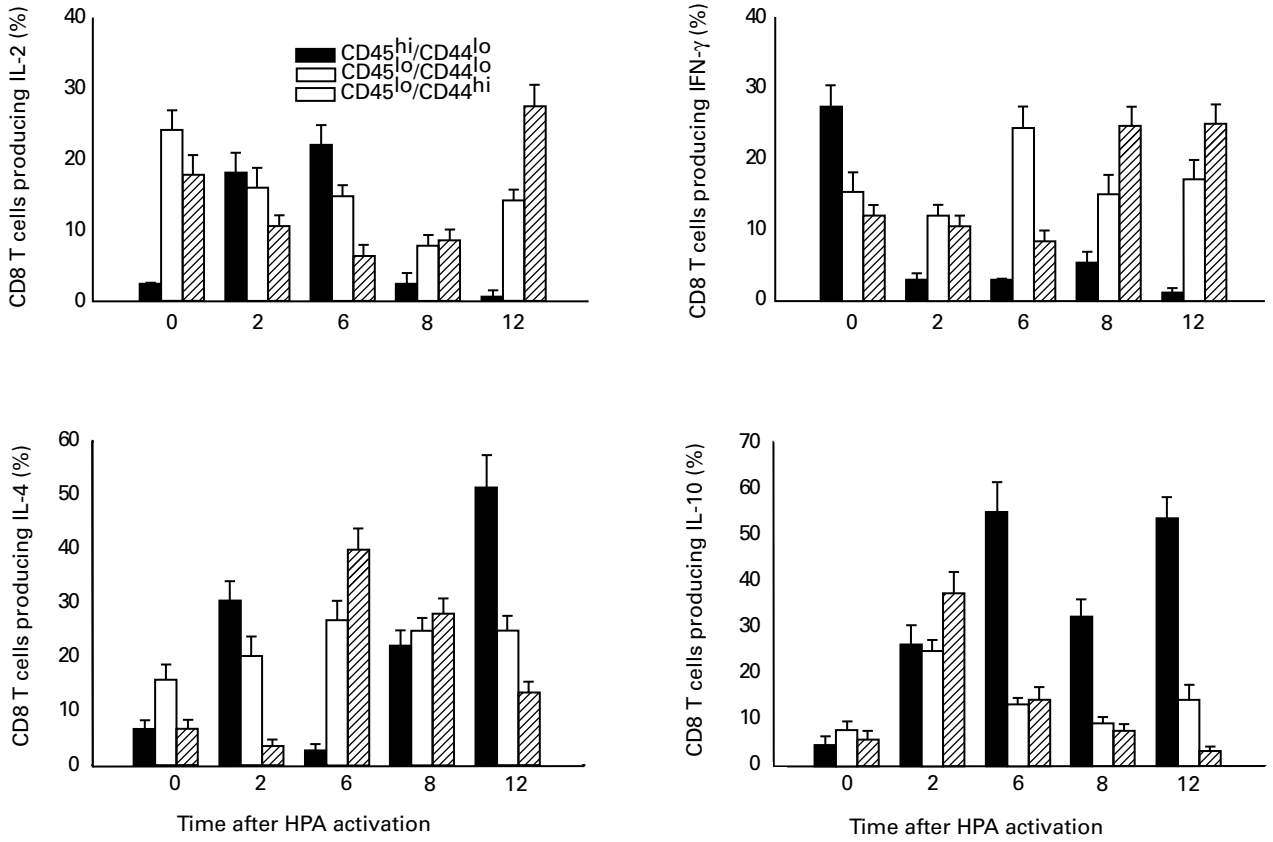

Fig. 3. Cytokine production by $CD8^+$ cells isolated from the lung of mice during reactivation of tuberculosis. The effect of hypothalamic–pituitary–adrenal (HPA) activation on the changes in cytokines of the different phenotypes of CD8 cells was significant for the $CD45^{hi}/CD44^{lo}$, $CD45^{lo}/CD44^{lo}$ and $CD45^{lo}/CD44^{hi}$ populations of cells ($P < 0{\cdot}001$).

[28,30,68,69]. The observation that memory CD4 cells were abundant in the lung within 6 weeks following initiation of HPA activation indicates that the cells differentiated along the Th1 pathway. While we did not determine if a Th0 population producing both IL-4 and IFN-γ was also present, the reappearance of Th1 cells indicates that sufficient levels of IFN-γ were present to cause the cells to differentiate along that pathway [28,30,68,69].

CD45 and CD44 phenotypes also distinguished a population of T0 cells. The number of CD4 Th0 cells expressing the $CD45^{lo}/CD44^{lo}$ phenotype produced both IFN-γ and IL-10 in the lung and increased following HPA activation. In contrast, the number of T0 CD4 and CD8 $CD45^{lo}/CD44^{hi}$ cells was low and remained unaffected by activation of the HPA axis and re-initiation of mycobacterial growth.

The results of this investigation provide the first insight into the changes that occur in T cell populations during the increase in mycobacterial growth that follows the activation of the HPA axis. Other cytokines, such as IL-18, IL-15 and IL-12 in addition to IFN-γ, have been shown to play an important role in the induction and maintenance of Th1 cells [61–66]. The changes in these cytokines, which are produced by macrophages and NK cells, as a result of HPA activation remain to be determined. While corticosterone has also been shown to suppress macrophage-mediated anti-mycobacterial activity *in vitro* [35,42], we believe that reactivation is due to an alteration in macrophage-activating cytokines rather than to a direct suppression of these important effector cells. Previous work by us has shown that activation of the HPA axis resulted in reactivation of *M. tuberculosis* growth in both Bcg^r and Bcg^s mice [43]. The anti-mycobacterial activity of macrophages from Bcg^r mice is not suppressed by corticosterone [35,42]. Thus, reactivation would appear to be the result of a lack of cytokines that maintain high levels of macrophage activation rather then a direct suppression of the macrophage.

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