Depletion of CD4+ T Cells Causes Reactivation of Murine Persistent Tuberculosis Despite Continued Expression of Interferon γ and Nitric Oxide Synthase 2

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Abstract

Tuberculosis is a major cause of death in much of the world. Current estimates are that one-third of the world’s population is infected with Mycobacterium tuberculosis. Most infected persons control the infection but in many cases may not eliminate the organism. Reactivation of this clinically latent infection is responsible for a large proportion of active tuberculosis cases. A major risk factor for reactivation of latent tuberculosis is HIV infection, suggesting a role for the CD4+ T cell subset in maintaining the latent persistent infection. In this study, we tested the requirement for CD4+ T cells in preventing reactivation in a murine model of latent tuberculosis. Antibody-mediated depletion of CD4+ T cells resulted in rapid reactivation of a persistent infection, with dramatically increased bacterial numbers in the organs, increased pathology in the lungs, and decreased survival. Although CD4+ T cells are believed to be a major source of interferon (IFN)-γ, expression of the gene for IFN-γ in the lungs of CD4+ T cell–depleted mice was similar to that in control mice. In addition, inducible nitric oxide synthase production and activity was unimpaired after CD4+ T cell depletion, indicating that macrophage activation was present even during CD4+ T cell deficiency. These data indicate that CD4+ T cells are necessary to prevent reactivation but may have roles in addition to IFN-γ production and macrophage activation in controlling a persistent tuberculous infection.

Key words: Mycobacterium tuberculosis • bacterial infection • macrophage • lung • nitric oxide synthase

Introduction

Mycobacterium tuberculosis, the etiologic agent of tuberculosis, was responsible for an estimated 8 million cases of tuberculosis and 1.5 million deaths worldwide in 1998 (1). In humans, the majority of M. tuberculosis infections are initially controlled, and a clinically latent infection is established, characterized by the persistence of low numbers of possibly dormant bacilli. A small but significant percentage (~10%) of latent infections reactivate years to decades later to give rise to reactivation tuberculosis (2). Aging (3) and iatrogenic immunosuppression (4) have been associated with reactivation of human latent infections. In recent years, HIV infection has emerged as the biggest risk factor for reactivation of latent tuberculosis (5). The severe CD4+ T cell deficiency in AIDS implicates CD4+ T cells in protection against reactivation tuberculosis. Cell-mediated immunity, contributed by both CD4+ and CD8+ T lymphocytes, plays an essential role in containing acute M. tuberculosis infection in murine models (for review see reference 6). Studies in mouse models using antibody depletion (7–9), adoptive transfer (10), and transgenic mouse strains deficient in either MHC class II (11, 12) or CD4 (12) have established the absolute requirement for CD4+ T cells in controlling an acute M. tuberculosis challenge. The key role of the CD4+ T cell in tuberculosis is thought to be its ability to produce the cytokine IFN-γ, which is essential in the control of experimental tuberculosis in mice (13, 14) and is the first identified human immuno-
nologic factor essential to resistance against mycobacterial infection (15). IFN-γ is a critical factor for inducing macrophage synthesis of the enzyme inducible nitric oxide synthase (NOS2) (14, 16). Upon activation, macrophages generate nitric oxide and other reactive nitrogen intermediates (RNIs), the best characterized antituberculous effector molecules in the mouse (for review see reference 17). Evidence is mounting that RNIs also play a role in antimycobacterial defense in humans (18). It is also likely that RNLI-independent mechanisms induced by IFN-γ participate in protection against tuberculosis (17, 19).

Despite the large body of knowledge on the immune response required to control an experimental acute M. tuberculosis infection, little is known about the immunologic mechanisms responsible for maintaining a latent infection. Studies using persistent tuberculosis in mice to model latent tuberculosis in humans have demonstrated that RNIs are required to prevent reactivation of persistent infection (19, 20). IFN-γ and TNF-α also participate in maintenance of persistent M. tuberculosis infection in mice (21, 22, and M ohan, V.P., C.A. Scanga, K. Yu, H.M. Scott, K.E. Tanaka, E. Tsang, J.L. Flynn, and J. Chan, manuscript submitted for publication). Although CD4+ T cells clearly are important early in the course of M. tuberculosis infection, the role of these cells beyond the acute phase of infection when a vigorous immune response has been established is unknown. CD4+ T cell-deficient mice succumbed to acute tuberculosis; although the level of IFN-γ was merely delayed compared with control mice, a compensatory increase in CD8+ T cells producing IFN-γ occurred by 4 wk after infection (12). This suggested that subsequent to the induction of an immune response to the infection, other cells might be capable of producing IFN-γ, reducing the requirement for CD4+ T cells.

In this study, we tested whether CD4+ T cells are required to prevent reactivation tuberculosis, using a previously described murine model of persistent tuberculosis (19). Depletion of CD4+ T cells resulted in marked reactivation of the infection. However, the expression of IFN-γ and NOS2 in the lungs of CD4+ T cell-depleted mice was similar to that in control mice, suggesting that the mechanism by which CD4+ T cells maintain a quiescent infection is not simply production of IFN-γ.

### Materials and Methods

**Mice.** 8-10-wk-old female C57BL/6 mice (The Jackson Laboratory and Charles River Laboratories) were housed in microisolator cages under specific pathogen-free biosafety level 3 conditions and monitored for various viruses, bacteria, and parasites. All animal protocols used in this study were approved by the Institutional Animal Care and Use Committees at Albert Einstein College of Medicine and the University of Pittsburgh School of Medicine.

**Mycobacteria and Infection.** The virulent Erdman strain (The Trudeau Institute, Saranac Lake, NY) of M. tuberculosis was passed through mice, grown once in culture, and frozen in aliquots. For infections, an aliquot was thawed, diluted in PBS with 0.05% Tween-80, and briefly sonicated in a cup horn sonicator, and 100 μl (containing 5 × 10^7 viable bacilli) was injected into mice via a lateral tail vein. The low-dose latency model, described previously (19), was used in this study. In brief, mice were infected with M. tuberculosis. Within 1 mo, the numbers of bacilli in the lungs and spleen reach 10^10–10^16 and the infection is stably maintained for >10 mo.

In *In Vivo* Depletion of CD4+ T Cells. 6–8 mo after infection, during the period of stable infection, CD4+ T cells were depleted in vivo using 0.5 μg of rat anti-CD4 mAb GK1.5 delivered intraperitoneally weekly (n = 20 mice per experiment). The GK1.5 hybridoma (ATCC) was used to produce ascites (Harlan Bioproducts for Science) and has been used by others for in vivo CD4+ T cell depletion (23). The ascites were subjected to sodium ammohnium sulfate precipitation to obtain CD4-specific IgG. Similarly infected control mice (n = 19 mice per experiment) received normal rat IgG (Jackson ImmunoResearch Laboratories). Mice that became moribund during the infection were humanely killed.

**CFU Determination.** Organs were homogenized in PBS/0.05% Tween-80 and dilutions were plated on 7H10 agar. Plates were incubated at 37°C in 5% CO2, and colonies were enumerated after 21 d.

**Histology and Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin for histological analysis and for acid fast bacilli using Kinyoun's method (Difco Labs.) according to the manufacturer's directions. Immunohistochemical staining for NOS2 was performed as described previously (19). In brief, formalin-fixed, paraffin-embedded tissue sections were deparaffinized, subjected to microwave antigen retrieval, and stained using rabbit anti-NOS2 antibody (Transduction Labs.), followed by biotinylated anti-rabbit IgG (Vector Labs.). Visualization of antibody for NOS2 was accomplished using an ABC kit (Vector Labs) and diaminobenzidine (DAB) or 3-amino-9-ethyl-carbazole substrate (Sigma-Aldrich) followed by a hematoxylin counterstain. Nitrotyrosine was detected in similarly treated tissue sections using a rabbit polyclonal antinitrotyrosine antibody (Upstate Biotechnology) with DAB substrate and a methyl green counterstain. Antibody specificity was confirmed by preincubating the primary antibody with 10 mM 3-nitro-l-tyrosine (Sigma-Aldrich) before incubation with the tissue sections.

**FACS Analysis.** To determine the efficacy of GK1.5 anti-CD4 mAb for CD4+ T cell depletion in vivo, single-cell suspensions were prepared by passing spleens harvested from long-term-infected mice treated with GK1.5 or normal rat IgG through mesh bags (Bally Ribbon Mills). RBCs were lysed with Tris/NaCl, and the cells were stained with mAbs directed against CD3, CD4, and CD8 (clones 145-2C11, H129.19, and 53-67, respectively; BD Pharmingen) at 0.2 μg/10^6 cells. The cells were fixed with 2% paraformaldehyde and subjected to three-color FACS® analysis using CELLQuest™ software on a FACSCalibur™ (Becton Dickinson). Cells were gated on lymphocytes by forward and side scatter parameters.

**Intracellular Cytokine Staining.** All antibodies used in FACS® analysis were obtained from BD Pharmingen. Single-cell suspensions of lymph nodes were prepared as above. Cells were stimulated overnight with anti-CD3 and anti-CD28 mAbs (clones 145-2C11 and 37.51, respectively) at 0.1 and 1.0 μg/ml, respectively. Mohnens (3 μM) was added for the final 6 h. Cells were then stained for the surface markers CD3 and CD8 as above, permeabilized with saponin, and stained for intracellular IFN-γ (anti-IFN-γ-PE mAb; clone X MG1.2). Cells were fixed with 1% paraformaldehyde and analyzed by three-color FACS®. Cells were gated on lymphocytes by forward and side scatter parameters.

### Abbreviations

- NOS, nitric oxide synthase; RNIs, reactive nitrogen intermediates; RPA, ribonuclease protection assay.
Ribonuclease Protection Assay. Total RNA was isolated from flash-frozen lungs using Trizol (GIBCO BRL) according to manufacturer's instructions, with an additional phenol-chloroform extraction before RNA precipitation. Gene expression was assessed using the RiboQuant™ ribonuclease protection assay (RPA) system (BD PharMingen) using customized template sets that included probes for NOS2, IFN-γ, IL-12p40, TNF-α, IL-1α, and IL-1β and the GAPDH and L32 housekeeping genes in one set and IL-4, IL-12p40, IL-10, IL-1α, IL-1β, IFN-γ, GAPDH, and L32 in another. Expression of cell surface marker genes was analyzed with the CD-1 RPA probe (BD PharMingen). Band intensities on the autoradiographs were quantitated by densitometry (Personal Densitometer SI; Molecular Dynamics), and the ratio of band intensity between the gene of interest and a housekeeping gene, either GAPDH or L32, was calculated.

Statistical Analysis. Where appropriate, values were tested for statistical significance by unpaired Student's t test using InStat (v.2.03; GraphPad Software). CFU values were subjected to log transformation before analysis. P values <0.05 were considered to be significant.

Results

Course of Chronic Persistent Infection after CD4⁺ T Cell Depletion. A murine model of latent human tuberculosis (19) was used to assess the contribution of CD4⁺ T cells to preventing reactivation. C57BL/6 mice were infected intravenously with 5 × 10⁵ CFU M. tuberculosis Erdman strain, and the infection was allowed to progress for 6–8 mo. As detailed previously (19), the bacillary burden in the lungs, spleen, and liver increased for 3–4 wk after infection. As a cellular immune response to the infection is established, the numbers of bacilli stabilize in the organs and remain essentially unchanged for at least 10 mo. During this period, the mice remain clinically healthy. This prolonged period of stable bacillary numbers in an apparently healthy animal may be best characterized as a persistent or chronic infection but can serve as a useful experimental model of human latent tuberculosis.

6 mo after infection, during the period of stable bacterial load, CD4⁺ T cells were depleted in vivo by weekly administration of rat anti-murine CD4 mAb GK1.5. Splenocytes were analyzed by FACS® to evaluate the efficacy of the depletion regimen 11 d after initiation of the antibody treatment. GK1.5-treated mice exhibited a 93% reduction in the number of splenic CD4⁺ T cells as compared with similarly infected control mice receiving normal rat IgG at this time point (1.7% CD4⁺ T cells [mean, two mice] versus 22.7%;...
The number of CD8+ T cells was unchanged in GK1.5-treated mice compared with control mice (13.4 versus 14.0%). The bacillary burden increased steadily in lung, liver, and spleen beginning as early as 2 wk after depletion of CD4+ T cells, while the numbers of bacilli in the organs of animals receiving normal rat IgG remained unchanged (Fig. 1 A). By 9 wk of antibody treatment, the GK1.5-treated mice had ~60-fold more mycobacteria in the lungs and spleen and 400-fold more in the liver compared with IgG-treated control mice (13.4 versus 14.0%). The bacillary burden increased steadily in lung, liver, and spleen beginning as early as 2 wk after depletion of CD4+ T cells, while the numbers of bacilli in the organs of animals receiving normal rat IgG remained unchanged (Fig. 1 A). By 9 wk of antibody treatment, the GK1.5-treated mice had ~60-fold more mycobacteria in the lungs and spleen and 400-fold more in the liver compared with IgG-treated control mice (13.4 versus 14.0%). The bacillary burden increased steadily in lung, liver, and spleen beginning as early as 2 wk after depletion of CD4+ T cells, while the numbers of bacilli in the organs of animals receiving normal rat IgG remained unchanged (Fig. 1 A). By 9 wk of antibody treatment, the GK1.5-treated mice had ~60-fold more mycobacteria in the lungs and spleen and 400-fold more in the liver compared with IgG-treated control mice (13.4 versus 14.0%).

In a second trial in which mice were infected for 8 mo before anti-CD4 treatment, the kinetics of tissue bacterial burden were virtually identical in GK1.5-treated mice (Fig. 1 B). CD4+ T cell–depleted mice exhibited a mean survival time of 76 ± 25 d with 100% mortality by 109 d of antibody treatment (range 46–109 d; n = 9 mice followed for mortality of 25 total). 4 of 25 control IgG–treated mice died during the antibody treatment period at 27, 35, 54, and 97 d of treatment. Although the bacillary burdens were not determined in the animals that died, analysis of other control mice indicated that bacterial numbers and pathology remained stable throughout the treatment period (Fig. 1 B). It is likely that the four deaths in the control IgG mice were not due to progression of tuberculosis but instead may have been caused by inadvertent penetration of visceral organs during antibody administration. In sum, depletion of CD4+ T cells was detrimental to control of chronic persistent tuberculosis.

Pathology in Persistently Infected Mice after CD4+ T Cell Depletion. Tissue sections from the CD4+ T cell–depleted and control mice were examined for histopathology. Not surprisingly, substantial pathology was present in lungs of both GK1.5- and control antibody–treated mice, as both groups had been persistently infected with M. tuberculosis for >6 mo. In control mice receiving normal rat IgG, the granulomatous response in the lungs consisted of well delineated lymphoid aggregates containing a minor monocytic component, situated among a predominately lymphohistiocytic infiltrate with poorly defined margins. There was an abundance of foamy macrophages, some of which were intraalveolar (Fig. 2, A and C). Alveolitis and interstitial pneumonitis were also observed. In the CD4+ T cell–depleted mice, a gradual disappearance of the lymphoid aggregates was apparent such that they were barely detectable microscopically by 63 d of GK1.5 treatment (Fig. 2, B and D). Most striking was the prominence of necrosis in the pulmonic granulomata of the CD4+ T cell–depleted mice, involving multiple areas with marked destruction of the lung architecture (Fig. 2 D), particularly at later time points when tissue bacillary burden was high. Eosinophilic plasma cells (Mott cells) were noted in granulomatous areas in the lungs of both the CD4+ T cell–depleted and control mice. These histopathologic findings are consistent with the increased CFU in organs of GK1.5–treated mice, in that high bacterial numbers are frequently associated with necrosis of lung tissue (24). Histologic stains for Pneumocystis carinii were negative (data not shown) and excluded this opportunistic pathogen as contributing to the histopathology observed in the CD4+ T cell–depleted mice.

![Figure 2. Histopathology in lungs of mice persistently infected with M. tuberculosis and then depleted of CD4+ T cells. Mice were infected with M. tuberculosis for 6 mo and then treated with GK1.5 monoclonal anti-CD4 or control IgG. Lungs were harvested 2 (top panels) or 9 wk (bottom panels) into the antibody regimens. Foamy alveolar macrophages (arrows) are apparent in both groups. Original magnification, 100×; insets, 400×. Micrographs from sections of representative mice are shown; sections from three to five mice per group per time point were examined, and similar results were obtained in a repeat experiment.](image-url)
**Table I.** Number of Cells Expressing NOS2 on Lung Sections of Persistently Infected Mice after Initiation of CD4+ T Cell Depletion Regimen

| Day 0 | 2 wk | 6 wk | 9 wk |
|-------|------|------|------|
| GK1.5 | IgG  | GK1.5 | IgG  | GK1.5 | IgG  |
| 70 ± 18 | 78 ± 17 | 101 ± 8 | 102 ± 11 | 114 ± 33 | 153 ± 25 | 181 ± 18 |

Beginning 6 mo after infection with M. tuberculosis, mice were treated with GK1.5 anti-CD4 antibody or control IgG. At the indicated time intervals after initiation of antibody treatment, lungs were recovered and immunohistochemically stained for NOS2 expression. The number of cells stained positively for NOS2 per 10^100^ fields ± SE is shown. Three to five mice per time point per group were examined.

NOS2 Expression and Activity in CD4+ T Cell-depleted Mice. The expression of NOS2 protein in lungs, spleen, and liver of CD4+ T cell-depleted and control mice was assessed immunohistochemically. High levels of NOS2 protein localized to foamy macrophages within granulomatous areas were observed at 6 mo after infection, before antibody treatment, as previously described (19; data not shown). There was no discernible difference in tissue NOS2 expression between GK1.5- and normal IgG-treated mice after 2 wk (Fig. 3, A and E), 6 wk (Fig. 3, B and F), or 9 wk (data not shown) of antibody treatment. The loss of CD4+ T cells did not appear to affect the number of NOS2-positive cells (Table I), the distribution of those cells, nor the intensity of NOS2 staining in the lung. This observation was supported by RPA analyses of total lung RNA from GK1.5 and control mice (Fig. 4). Expression of the NOS2 gene was similar between CD4+ T cell-depleted and control mice at all time points. More direct evidence of continued production of RNIs in the CD4+ T cell-depleted mice was provided by immunostaining tissue sections from these mice using antinitrotyrosine antibody to detect nitrated proteins. Nitrotyrosine is generated via nitration of tyrosine by peroxynitrite, a product of NO and superoxide anion, and thus reflects NOS2 activity. Nitrotyrosine staining was similar in the lung tissue and granulomas of GK1.5- and normal IgG-treated mice (Fig. 3, C and H). When the antinitrotyrosine antibody was preincubated with nitrotyrosine before being used in this assay, all staining was abolished (Fig. 3, D and G), confirming the staining specificity. These results indicate that CD4+ T cell depletion-induced reactivation was not due to a deficiency in RNI-dependent antimicrobial mechanisms.

**Figure 3.** NOS2 protein expression and protein nitration in lungs of mice persistently infected with M. tuberculosis for 6 mo and then depleted of CD4+ T cells. 6 mo after infection, mice were treated with GK1.5 monoclonal anti-CD4 to deplete CD4+ T cells (E–H) or control IgG (A–D). Lung sections were stained for NOS2 protein (A, B, E, and F) 2 wk (A and E) and 6 wk (B and F) into the antibody treatment regimen. Similarly processed lung sections obtained 6 wk into the antibody regimen were also stained for nitrotyrosine (C and G). Specificity of the nitrotyrosine antibody was confirmed by preincubating the nitrotyrosine antibody with nitrotyrosine before staining the sections (D and H). B–D are serial sections, as are F–H. Representative sections from four mice per group per time point are shown. Original magnification, 400×.
IFN-γ Production after CD4+ T Cell Depletion. IFN-γ is a critical factor required for activating macrophages to produce NOS2 (14, 16). Therefore, the finding of unimpaired NOS2 production in CD4+ T cell–depleted mice suggested that an alternate source of IFN-γ, independent of CD4+ T cells, existed in GK1.5-treated mice. To address the possible source of IFN-γ in the lungs of the mice, cell populations and cytokine production in the lungs were assessed by FACS® analysis. Staining for CD4+ T cells confirmed that GK1.5 treatment was efficacious in the lungs, with >94% depletion of CD4+ T cells in lungs of GK1.5-treated mice compared with normal rat IgG–treated mice after 4 and 9 wk of antibody treatment (Fig. 5). However, the mean number of cells recovered from lung tissue did not differ significantly between GK1.5- and normal rat IgG–treated mice (Table II). There was an increase in the percentage of CD8+ T cells in CD4+ T cell–depleted mice compared with control IgG–treated mice at both 4 wk (mean GK1.5, 20.5%; mean control IgG, 10.8%; P = 0.03) and 9 wk (mean GK1.5, 34.1%; mean control IgG, 26.9%; P = 0.076). Because the numbers of cells recovered from the lungs of mice from both groups were similar (Table II), this increased percentage corresponds to an actual increase in total number of CD8+ T cells.

We used intracellular cytokine staining of lung cells to assess production of IFN-γ in the lungs of the CD4+ T cell–depleted, persistently infected mice. Despite effective depletion of the pulmonic CD4+ T cell compartment, there was not a significant decrease in the percentage of total lymphocytes producing IFN-γ after 4 or 9 wk of depletion compared with IgG-treated control mice. Data from representative mice are shown in Fig. 5. Lymphocytes from uninfected mice produce very little IFN-γ after ex vivo stimulation (25). As noted above, there was an increase in numbers of CD8+ T cells in GK1.5-treated mice, and these CD8+ T cells were capable of producing IFN-γ (Fig. 5).

RPA analysis of total lung RNA confirmed that the levels of IFN-γ expression in the lungs of GK1.5– and control IgG–treated mice were indeed comparable (Fig. 4). The efficacy of GK1.5 treatment, the increase in CD8+ T cells...
upon CD4\(^+\) T cell depletion, and the comparable IFN-\(\gamma\) expression in the lungs of GK1.5 and control mice was confirmed in a second CD4\(^+\) T cell depletion experiment (data not shown). Taken together, these data indicate that in the lungs of persistently infected mice depleted of CD4\(^+\) T cells, there was an increase in CD8\(^+\) T cells capable of IFN-\(\gamma\) production.

**Gene Expression in the Lungs after CD4\(^+\) T Cell Depletion in Chronic Persistent Tuberculosis.** RPA analysis of total lung RNA revealed that in the CD4\(^+\) T cell–depleted mice, TNF-\(\alpha\) expression remained relatively steady, except at the last time point when a significant increase was observed (Fig. 4). This may reflect the more severe pathology in the lungs of these mice relative to the mice receiving normal rat IgG (Fig. 2). Substantial expression of IL-1\(\alpha\) and IL–1\(\beta\) was detected over the course of the antibody treatment regimen, but there was little difference between the mice receiving GK1.5 and those receiving control antibody (Fig. 4). A low level of IL-12p40 expression was noted in both groups and did not vary throughout the antibody treatment period (data not shown). Expression of IL-4 (Fig. 4) and IL-10 (data not shown) was low or undetectable at all time points in both groups of mice.

2 wk after initiation of anti-CD4 antibody treatment, there was a decrease in CD4 gene expression in the lungs relative to that in mice receiving control antibody, as assessed by RPA (Fig. 6). This difference was significant (\(P = 0.002\)) by 6 wk of antibody treatment and was maintained throughout the remainder of the experiment (Fig. 6). These data confirmed the results obtained by FACScan analysis that GK1.5 was efficacious in depleting CD4\(^+\) T cells in the lungs over a long treatment period. The expression of CD3 was not significantly different among the GK1.5- and rat IgG–treated mice at the various time points studied (Fig. 6). There was a significant decrease in the expression of CD19 (a pan-B cell marker) in CD4\(^+\) T cell–depleted mice by 6 wk of GK1.5 treatment compared with IgG–treated mice (\(P = 0.001\); Fig. 6), suggesting that fewer B cells were present in those mice.

### Discussion

The association between the deficiency of CD4\(^+\) T cells observed with AIDS and the incidence of reactivation of latent tuberculosis suggests that CD4\(^+\) T cells play an important role in preventing reactivation tuberculosis. In human studies, \(M.\) tuberculosis–specific CD4\(^+\) T cells secrete IFN-\(\gamma\) (26), and a recent publication suggests that in vitro IFN-\(\gamma\) can induce human macrophages to kill \(M.\) tuberculosis coincident with the expression of NOS2 (27). CD4\(^+\) T cells are thought to be the primary source of IFN-\(\gamma\) and are required to control acute \(M.\) tuberculosis infection in mice (7, 8, 10, 12). The importance of IFN-\(\gamma\) in controlling mycobacterial infections has been demonstrated in humans.
The requirement for CD4+ T cells in preventing reactivation was tested in a murine model of persistent tuberculosis in which CD4+ T cells were depleted by antibody treatment beginning 6-8 mo after infection. Depletion of CD4+ T cells resulted in fatal exacerbation of the quiescent infection with markedly increased bacterial numbers, indicating that CD4+ T cells are required to prevent reactivation. Two surprising findings emerged from this study. First, depletion of CD4+ T cells, arguably the major source of IFN-γ in tuberculous infection, did not result in an appreciable decrease in overall IFN-γ production in the lungs. Second, control of mycobacterial growth was lost in the CD4+ T cell-depleted mice, despite unimpaired NOS2 expression and activity.

CD8+ T cells are capable of producing significant amounts of IFN-γ in response to M. tuberculosis in both murine (11, 12, 25) and human (28, 29) systems. Indeed, in this study, depletion of CD4+ T cells in mice persistently infected with M. tuberculosis resulted in an increase in the number of CD8+ T cells, a subset of which was producing IFN-γ. Therefore, as in acute tuberculosis models using CD4+ T cell-deficient mice (12), a compensatory increase in CD8+ T cells producing IFN-γ in the lungs of GK1.5-treated mice could at least partially account for the diminished IFN-γ expression seen in mice depleted of CD4+ T cells. Other cell types cannot be excluded from contributing to IFN-γ production. CD4+8− T cells from Leishmania major-infected mice have been shown to produce IFN-γ in vitro (30), although this was not observed in acute tuberculosis models in CD4+ T cell-deficient mice (12). NK cells have been implicated as important sources of IFN-γ in the early immunologic response against several infectious agents, including L. major (31) and Toxoplasma gondii (32). Finally, macrophages have been reported to produce IFN-γ in response to mycobacteria (33, 34). As cell markers other than CD8 and CD4 were not analyzed by FACS® contribution to the total IFN-γ production by these other cell types cannot be excluded. Whatever the source of the IFN-γ, it is clear that its presence did not prevent reactivation of the infection and thus did not adequately compensate for the lack of CD4+ T cells. Perhaps this cytokine must be delivered directly to the infected macrophage to be effective, and cell types other than CD4+ T cells may not do so as efficiently within the granuloma. Studies are underway to determine the precise location of and the cell type responsible for IFN-γ production in the absence of CD4+ T cells in this model.

Studies using murine models of latent tuberculosis have established the importance of NOS2 in preventing reactivation (19, 20). In this study, however, CD4+ T cell depletion resulted in a dramatic increase in the numbers of bacilli despite levels of NOS2 comparable to that of mice receiving control IgG. Nitrated tyrosine residues were detected in lung tissue of both CD4+ T cell-depleted and control mice, and their distribution was colocalized to areas of NOS2 expression. These data provide evidence that NOS2 gene and protein expression and activity were unaffected by the depletion of CD4+ T cells. NOS2 expression is de-

Figure 6. Changes in lung cell composition after GK1.5 anti-CD4 treatment of mice persistently infected with M. tuberculosis. Mice infected with M. tuberculosis for 6 mo were injected with GK1.5 (open symbols) or control IgG (closed symbols), and lung gene expression was measured by RPA as described in Fig. 4 using a template specific for cell markers including CD4 (top), CD3 (center), and CD19 (bottom). Each point represents the mean of results obtained from three to five mice, and the bars are SE. *P < 0.05. Similar results were obtained in a repeat experiment.

(for review see reference 15) and mice (13, 14). NOS2 expression is severely compromised at both the gene (14) and protein (our unpublished observations) levels in M. tuberculosis-infected mice with a disruption in the IFN-γ gene, and these animals succumb quickly to infection (14). In addition, emerging evidence suggests that both IFN-γ (22) and NOS2 (19, 20) play a role in preventing reactivation of persistent tuberculosis in murine models of latency.
In light of reactivation in the presence of control levels of IFN-γ and NOS2 in the CD4+ T cell-depleted mice, additional CD4+ T cell functions capable of maintaining latency must be considered. First, the requirement of CD4+ T cells for a vigorous granulomatous reaction in mycobacterial infection has been reported (12, 36, 37). In tuberculosis patients coinfected with HIV, the structural integrity of the tuberculous granuloma appears to correlate with the number of total peripheral CD4+ T cells (38, 39).

Although the pathology and general loss of granuloma structure observed in the lungs of GK1.5-treated mice during reactivation may be directly related to bacterial numbers, we cannot exclude a role for CD4+ T cells in maintenance of an organized granuloma during latent infection. Second, a key element in controlling M. tuberculosis infection is macrophage activation. CD4+ T cells may have IFN-γ- and NOS2-independent pathways to activate mycobactericidal or mycobacteriostatic effector functions in macrophages. Macrophages can be activated through direct contact via interaction between CD40 on macrophages and CD40L on activated lymphocytes (40), and this can result in NOS2 induction (41). Although this mechanism is apparently necessary for successful immunity to L. major (42–44), CD40L−/− mice were not more susceptible to M. tuberculosis infection (45). As negative data in genetically deficient mice can be difficult to interpret, the role of CD40 in tuberculosis remains unclear. Third, it is possible that depletion of CD4+ T cells results in increased production of cytokines capable of deactivating macrophages. Two such cytokines are IL-10 (46) and TGF-β (47). Indeed, in HIV-infected individuals, production of IL-10 and TGF-β by PBMCs is augmented (for review see reference 48). IL-10 mRNA was expressed at very low levels that were similar in lungs of CD4+ T cell-depleted and control mice, so this cytokine is not likely to contribute to the reactivation observed in this model. TGF-β produced by M. tuberculosis-infected macrophages has been reported to downregulate macrophage function (49). TGF-β was not examined in this study, and so the possibility remains that this cytokine may increase in the absence of CD4+ T cells, leading to macrophage deactivation and increased bacterial growth. Fourth, CD4+ T cells are thought to be important in maintaining an adequate CD8+ T cell response (50–52). We and others have demonstrated previously that CD8+ T cells participate in control of tuberculosis in mice (9, 11, 25, 53, 54), and the presence of mycobacterial-specific CD8+ T cells in tuberculosis patients has been reported (28, 29, 55). Mycobacteria-specific CD8+ T cells can produce IFN-γ or act as cytotoxic cells for infected macrophages (25, 28, 29, 56, 57). Recent data indicate that CD8+ CTLs that produce granulysin can kill intracellular M. tuberculosis (58). Although CD8+ T cell numbers were increased after CD4+ T cell depletion, and these cells were clearly primed to produce IFN-γ, the actual function of these cells in the lungs of the absence of CD4+ T cells is unclear. Perhaps CD4+ T cells are necessary for complete function or maintenance of CD8+ cytolytic activity, without which control of chronic persistent tuberculosis is compromised.

Results obtained from RPA analysis of cell surface marker expression suggested that there was a substantial reduction in B cells in the lungs of CD4+ T cell-depleted mice. T cells play an important role in modulating B cell differentiation, function, and lifespan. CD4+ T cells may also regulate production of specific B cell chemoattractants. Plasmacytoid cells were observed in lung sections, but CD19 is downregulated on fully differentiated B cells in humans (59, 60), and so this does not contradict the RPA data. The role of antibodies in immunity to M. tuberculosis is controversial (61), and B cells may contribute to the immune response to intracellular infections in an antibody-independent manner, as suggested recently in a Francisella tularensis murine model (62). Studies using B cell–deficient mice are contradictory, suggesting that B cells contribute modestly (63) or not at all (64) to control of M. tuberculosis infection. A careful analysis of the contribution of B cells in latent tuberculosis may further define protective mechanisms against reactivation of latent infections.

In this murine model of latent tuberculosis, inhibition of NOS2 (19), neutralization of TNF-α (Mohan, V.P., C.A. Scanga, K. Yu, H.M. Scott, K.E. Tanaka, E. Tsang, J.L. Flynn, and J. Chan, manuscript submitted for publication), or depletion of CD4+ T cells (this study) resulted in reactivation of infection and fatal tuberculosis. However, there were significant differences in the course of reactivation and pathology as well as expression of cytokines, depending on the immunologic manipulation implemented. Inhibition of NOS2 resulted in slowly progressive reactivation primarily in the lungs (19), whereas CD4+ T cell depletion resulted in a rapid and dramatic increase in bacterial numbers in liver and spleen, as well as lung, shortly after initiation of antibody treatment. TNF-α neutralization caused...
an initial increase in bacterial numbers that stabilized after 3 wk, despite decreased NOS2 expression (Mohan, V.P., C.A. Scanga, K. Yu, H.M. Scott, K.E. Tanaka, E. Tsang, J.L. Flynn, and J. Chan, manuscript submitted for publication). Severe pathology was observed in TNF-α–neutralized mice that was very different than that observed in CD4+ T cell–depleted mice, with marked disorganization of granulomata and increased leukocytic infiltration (Mohan, V.P., C.A. Scanga, K. Yu, H.M. Scott, K.E. Tanaka, E. Tsang, J.L. Flynn, and J. Chan, manuscript submitted for publication). Although interpretations based on interexperimental comparisons must be made with prudence, the results of our previous studies and results presented in this study suggest that individual immunologic components play a variety of roles in preventing reactivation, highlighting the complex nature of the protective response in chronic tuberculosis.

In summary, this study demonstrates the requirement for CD4+ T cells in maintaining a persistent M. tuberculosis infection. However, depletion of CD4+ T cells did not result in decreased tissue expression of IFN-γ or NOS2, the two most characterized CD4+ T cell–related antimycobacterial factors. Thus, the role of the CD4+ T cell in persistent tuberculosis is more complex than merely as a source of IFN-γ for macrophage activation. Further investigation of the mechanism by which the CD4+ T cell functions during latency may guide the development of therapies and vaccines designed to prevent reactivation of tuberculosis.

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