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Mice Deficient in CD4 T Cells Have Only Transiently Diminished Levels of IFN-γ, Yet Succumb to Tuberculosis[1]

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CD4 T cells are important in the protective immune response against tuberculosis. Two mouse models deficient in CD4 T cells were used to examine the mechanism by which these cells participate in protection against *Mycobacterium tuberculosis* challenge. Transgenic mice deficient in either MHC class II or CD4 molecules demonstrated increased susceptibility to *M. tuberculosis*, compared with wild-type mice. MHC class II$^{-/-}$ mice were more susceptible than CD4$^{-/-}$ mice, as measured by survival following *M. tuberculosis* challenge, but the relative resistance of CD4$^{-/-}$ mice did not appear to be due to increased numbers of CD4^{-}8^{-} (double-negative) T cells. Analysis of in vivo IFN-γ production in the lungs of infected mice revealed that both mutant mouse strains were only transiently impaired in their ability to produce IFN-γ following infection. At 2 wk postinfection, IFN-γ production, assessed by RT-PCR and intracellular cytokine staining, in the mutant mice was reduced by >50% compared with that in wild-type mice. However, by 4 wk postinfection, both mutant and wild-type mice had similar levels of IFN-γ mRNA and protein production. In CD4 T cell-deficient mice, IFN-γ production was due to CD8 T cells. Thus, the importance of IFN-γ production by CD4 T cells appears to be early in infection, lending support to the hypothesis that early events in *M. tuberculosis* infection are crucial determinants of the course of infection. ***The Journal of Immunology*, 1999, 162: 5407–5416.**

Mycobacterium tuberculosis is responsible for approximately 3 million deaths/yr worldwide (1). This bacterium is primarily transmitted through the respiratory route and causes active tuberculosis in 10–15% of infected persons. The host response to the organism is a major determinant of the outcome of the infection, and cell-mediated immunity is required to prevent active disease. Studies in murine models have implicated both CD4 and CD8 T cells in protection against *M. tuberculosis* (reviewed in Ref. 2). Humans infected with HIV are strikingly more susceptible to both initial infection with *M. tuberculosis* and reactivation of latent infection (3, 4), implicating functional CD4 T cells in the control of human tuberculosis.

M. tuberculosis replicates within host macrophages, and activated macrophages are essential to limiting the infection. Macrophages activated by IFN-γ and either TNF-α or bacterial products such as LPS or lipoarabinomannan produce reactive nitrogen intermediates (RNI)[4] and kill intracellular mycobacteria (5–7). RNI produced by activated murine macrophages are required in vivo (8–10) and in vitro (6, 7) to control *M. tuberculosis* infection. We and others have demonstrated an absolute requirement for IFN-γ (11, 12) and TNF-α (13) in the control of murine tuberculosis, which is in part related to the requirement for these cytokines in early RNI production in vivo.

CD4 T cells are thought to be the major source of IFN-γ during *M. tuberculosis* infection, and it is generally believed that the primary role of CD4 T cells in controlling tuberculosis is production of this cytokine. Previous studies have shown that both murine and human *M. tuberculosis*-specific CD4 T cells produce IFN-γ (14–17) and can activate macrophages to kill *M. tuberculosis* in vitro (18). Mycobacterium-specific human CD8 T cells can also produce IFN-γ and lyse infected cells (19–21). Previous murine studies relied on Ab depletion in vivo or adoptive transfer of T cell subsets to show that CD4 T cells were involved in protection against *M. tuberculosis* (22–24). More recently, MHC class II-deficient mice were shown to have increased susceptibility to *M. tuberculosis* infection (25). Here, we have compared transgenic mice with defects in the expression of either MHC class II (26) or CD4 (27) molecules to test the requirement for CD4 T cells in *M. tuberculosis* infection and to assess the mechanism by which these CD4 T cells are protective against tuberculosis. We demonstrate that IFN-γ production by both CD4 T cell-deficient mouse strains was only transiently reduced, yet the mice succumbed to the infection. These mice may serve as a useful model for AIDS and tuberculosis.

Materials and Methods

Mouse strains

All mice were bred in the specific pathogen-free facility at the University of Pittsburgh School of Medicine (Pittsburgh, PA). Breeding pairs for MHC class II$^{-/-}$ mice (26) were obtained from Dr. Diane Mathis (Strasbourg, France) and backcrossed four times onto the C57BL/6 background, and mice were bred as heterozygotes (+/− × −/− or +/− × +/−). To identify mice for use as breeders, mice were genotyped by Southern blot as previously described (26). Before use in experiments, each mouse was phenotyped by staining PBL with Ab against CD4 and analyzing by flow cytometry; mice lacking MHC class II molecules had very low levels of

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[4] Abbreviations used in this paper: RNI, reactive nitrogen intermediates; BCG, Calmette-Guérin bacillus; PPD, purified protein derivative; QC-RT-PCR, quantitative competitive RT-PCR; HPRT, hypoxanthine phosphoribosyltransferase.

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CD4 T cells compared with wild-type mice. Mice heterozygous or homozygous for the wild-type MHC class II gene (+/+ or +/−) were indistinguishable in response to *M. tuberculosis* (data not shown), so littermates (usually +/−) were used as control mice. $CD4^{-/-}$ breeding pairs were obtained from Dr. Tak Mak, backcrossed twice onto the C57BL/6 background, and bred as homozygotes. As controls, +/+ littermates were bred. Mice were phenotyped by staining PBL with anti-CD4 Ab and analyzing by flow cytometry to assess the presence or the absence of the CD4 molecule.

Bacteria and infections

M. tuberculosis (Erdman strain, Trudeau Institute, Saranac Lake, NY) was passed through mice, grown in culture once and frozen in aliquots. Before injection into mice, an aliquot was thawed, diluted in PBS containing 0.05% Tween-80, and sonicated for 10 s in a cup-horn sonicator. Mice were infected i.v. via the tail vein with 2–10 $\times$ 10^5 (depending on the experiment) live bacilli in 100 μl, as determined by viable counts on 7H10 agar plates (Difco, Detroit, MI). For immunization, Calmette-Guérin bacillus (BCG; Pasteur strain, Trudeau Institute; 1 $\times$ 10^5 live bacilli i.v.) and *M. tuberculosis* (1 $\times$ 10^3 live bacilli i.v.) were administered to mice. In some experiments mice were treated 4 wk after immunization with isoniazid (Sigma, St. Louis, MO; 1 mg/ml in drinking water) for 4 wk. Ten days following the end of antibiotic treatment, the mice were challenged with virulent *M. tuberculosis* as described above.

CFU determination

Organs retrieved from infected mice were homogenized in PBS/Tween-80 (0.05%) in plastic bags using a Stomacher homogenizer (Tekmar, Cincinnati, OH), and dilutions were plated on supplemented 7H10 agar plates. Colonies were counted after 21-day incubation at 37°C in 5% CO_2.

Histology

Organs were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin or with Kinyoun's stain for acid-fast bacilli. The numbers of granulomas in liver sections were counted in 5–10 $\times$10 fields from each slide, using hematoxylin- and eosin-stained sections (three or four mice per group). For immunohistochemistry, paraffin-embedded sections were deparaffinized, and Ag retrieval was performed using a microwave technique as previously described (8). Anti-NOS2 Ab (Transduction Laboratories, Cincinnati, OH) was used to stain tissues, with biotinylated anti-rabbit IgG as a secondary Ab. The ABC method (Vector, Burlingame, CA) was used with diaminobenzidene as a substrate to visualize Ab binding. The proportion of $NOS2^+$ granulomas was assessed by counting total granulomas versus $NOS2^+$ granulomas in 5–10 $\times$10 fields of liver sections from each mouse.

FACS analysis of cell surface markers

Spleen cells were obtained from infected mice at various time points postinfection by crushing the organ in mesh bags to obtain single cell suspensions. RBC were lysed with Tris/NH_4Cl solution. Cells were stained for cell surface molecules using Abs against CD3 (anti-CD3-phycoerythrin), CD4 (anti-CD4-FITC), and CD8 (anti-CD8-CyChrome). All Abs were used at 0.2 μg/10^6 cells and were obtained from PharMingen (San Diego, CA). Cells were fixed with 2% paraformaldehyde overnight and analyzed by FACS (Becton Dickinson, Mountain View, CA). Two or three mice per group were used for each time point.

Spleen cell proliferation assays

Single cell suspensions were obtained by crushing spleens in mesh bags. RBC were lysed with Tris/NH_4Cl, and the cells were washed extensively. Following resuspension in medium (RPMI 1640, 10% FBS, glutamine, and 2-ME), 5 $\times$ 10^5 cells/well of 96-well round-bottom plates were stimulated with medium alone, Con A (5 μg/ml), or PPD (10 μg/ml). In some experiments 1 $\times$ 10^4 peritoneal exudate macrophages from C57BL/6 mice were added to the wells before addition of spleen cells. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) after 60 h of culture and were harvested 12–18 h later. Incorporation of [^{3}H]thymidine was measured by counting cell lysates on filters in a scintillation counter. The stimulation index was determined by dividing sample counts by background counts for each sample and is presented as an average for two mice per group. The experiment was repeated three times.

ELISA

IFN-γ production by cultured spleen cells was assessed by sandwich ELISA using Abs R4-A62 and XMG1.2 (biotinylated; PharMingen), according to the manufacturer's protocol. Recombinant murine IFN-γ, used to generate a standard curve, was a gift from Genentech (South San Francisco, CA).

Semiquantitative competitive RT-PCR

Cytokine and NOS2 mRNA levels in lung and spleen were assessed using the QC-RT-PCR method as previously described (8, 28). PCR products were electrophoresed on 2% agarose gels followed by staining with Sybr Green (Molecular Probes, Eugene, OR). Bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The concentration of cDNA in each sample was standardized by determining the ratio of lung cDNA to competitor plasmid DNA for hypoxanthine phosphoribosyltransferase (HPRT), using a fixed concentration of competitor plasmid. A correction value for each sample for standardization was obtained. The ratio of lung cDNA to competitor plasmid DNA for each cytokine was obtained and was corrected using the HPRT values for each sample. Relative levels of cytokine mRNA are reported (lung cDNA/plasmid DNA), and a value of 1 is equivalent to HPRT mRNA level. Time zero indicates uninfected mice. The data presented are an average of three or four mice per group per time point.

In vivo intracellular cytokine staining

Single cell suspensions of lung and spleen cells at various time points postinfection were prepared as described above. Cells were stimulated for 6 h with anti-CD3 (clone 145-2C11; 0.1 and anti-CD28 (clone 37.51; 1 μg/ml) Abs in the presence of 3 μM monensin (Sigma) to halt egress of cytokines from the cells. Following washing, cells were stained for cell surface molecules CD4 (0.2 μg/10^6 cells anti-CD4-CyChrome Ab, clone H129.19) and CD8 (0.2 μg/10^6 cells anti-CD8-FITC Ab, clone 53-6.7) in 20% mouse serum/1% FBS for 30 min at 4°C, washed, and fixed in 1% paraformaldehyde at 4°C overnight. Cells were permeabilized with saponin (0.1% in PBS containing 1% FBS/0.1% sodium azide) and were stained for IFN-γ or IL-4 (0.4 μg/10^6 cells, anti-IFN-γ-PE Ab, clone XMG1.2, or anti-IL-4-PE Ab, clone 11B11) in 20% mouse serum for 30 min at 4°C, washed, and analyzed by FACS (Becton Dickinson). Isotype controls for each Ab were used, and an uninfected control mouse was tested in each experiment. All Abs were obtained from PharMingen. Cells could not be stained with anti-CD3 Ab for analysis, as anti-CD3 Ab was used to stimulate the cells.

Statistics

Student's paired test was used to compare groups. Statistical analysis was performed using StatView (Abacus Concepts, Berkeley, CA).

Results

Course of infection in CD4 T cell-deficient mice

Mice deficient in MHC class II molecules (26) were infected with virulent *M. tuberculosis* (5 $\times$ 10^5 bacilli i.v.). These mice succumbed to the infection with a mean survival time of 39 $\pm$ 1 days, while control mice (homozygous +/+ or heterozygous +/− littermates) survived for the length of the experiment (>120 days; $p < 0.0001$; Fig. 1). The MHC class $II^{-/-}$ mice were deficient in their ability to control the infection in lungs, liver, and spleen compared with the control mice (Fig. 2). Whereas wild-type or heterozygous mice began to control the infection in lungs at 3 wk postinfection, MHC class $II^{-/-}$ mice did not, and at 40 days postinfection there was a 50-fold increase in number of bacteria in the MHC class $II^{-/-}$ mice compared with the controls. In spleen and liver, mutant mice had >100-fold higher bacterial numbers compared with control mouse organs. Thus, the absence of MHC class II molecules prevented the mice from controlling *M. tuberculosis* infection.

Although MHC class $II^{-/-}$ mice are deficient in CD4 T cells due to the lack of positive selection in the thymus, they are not completely lacking in these cells (Table III). As a second model, mice in which the gene CD4 molecule was disrupted ($CD4^{-/-}$) (27) were tested in our acute tuberculosis model. The mean survival time for $CD4^{-/-}$ mice infected with *M. tuberculosis* (5 $\times$ 10^5 bacilli i.v.) was 60 $\pm$ 7 days, which is significantly longer than that for MHC class $II^{-/-}$ mice (Fig. 1; $p = 0.02$). Again, controls

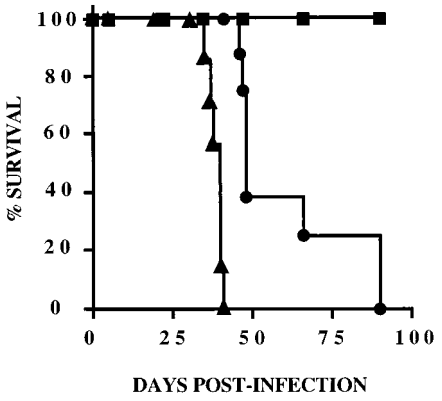

FIGURE 1. Survival of MHC class II- and CD4-deficient mice following *M. tuberculosis* infection. MHC class $II^{-/-}$ (▲), $CD4^{-/-}$ (●), and wild-type littermate (■) mice were infected i.v. with 5×10^5 viable *M. tuberculosis* bacilli (strain Erdman). Mean survival times were 39 ± 1 days for MHC class $II^{-/-}$ mice, 60 ± 7 days for $CD4^{-/-}$ mice, and >120 days for wild-type mice. At least eight mice per group were used. This is a representative experiment of three performed.

(+/+ littermates) survived the length of the experiment (>120 days). Bacterial numbers in lung, liver, and spleen were at least 100-fold greater in $CD4^{-/-}$ mice than in control mice by 35 days postinfection (Fig. 2).

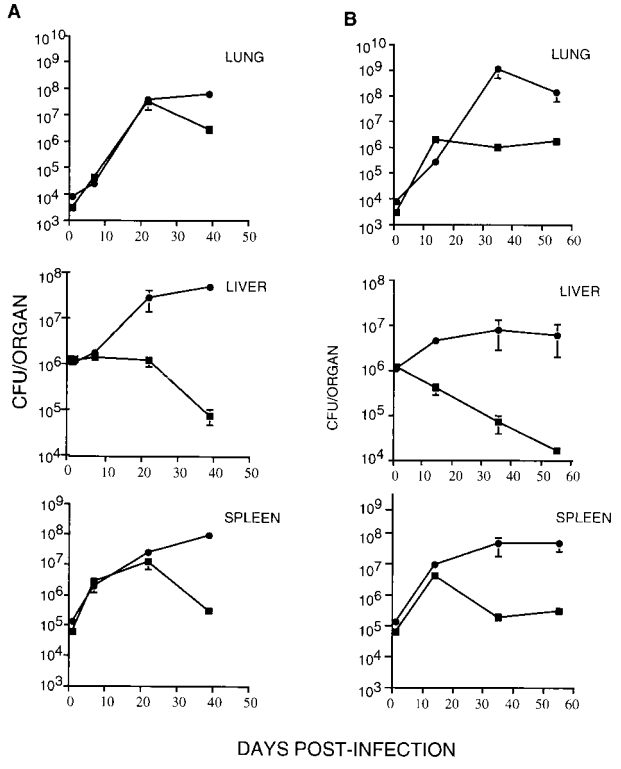

FIGURE 2. Bacterial burdens in the organs of wild-type, MHC class $II^{-/-}$, and $CD4^{-/-}$ mice following *M. tuberculosis* infection. *A*, MHC class $II^{-/-}$ (●) and wild-type littermate control (■) mice. *B*, $CD4^{-/-}$ (●) and wild-type control (■) mice. Mice were infected i.v. with 5×10^5 *M. tuberculosis* bacilli. Mice were sacrificed at various time points postinfection, and viable bacteria in lungs, livers, and spleens were determined by plating homogenate dilutions on 7H10 plates and counting colonies after incubation for 21 days. Four mice per group per time point were used; error bars indicate the SE. This is a representative experiment of four performed.

Table I. *Vaccination and challenge of MHC class $II^{-/-}$ mice*[a]

Mouse Strain	Immunization	Challenge (*M. tuberculosis*)	Mean Survival Time (days)
Wild type	None	5×10^5	>120
MHC class $II^{-/-}$	None	5×10^5	41 ± 1
Wild type	BCG	5×10^5	>120
Wild type	*M.tb* + INH	5×10^5	>120
MHC class $II^{-/-}$	BCG	5×10^5	43 ± 2
MHC class $II^{-/-}$	BCG + INH	5×10^5	39 ± 7
MHC class $II^{-/-}$	*M.tb* + INH	5×10^5	38 ± 2

[a] Mice were immunized i.v. with BCG or *M. tuberculosis (M.tb)*, and in some cases treated with isoniazid (INH) for 4 wk prior to challenge with virulent *M. tuberculosis*. A total of 6–10 mice per group were used.

BCG immunization in the absence of MHC class II molecules

Prior BCG immunization of immunocompetent mice does not prevent infection with a virulent *M. tuberculosis* challenge, but the bacterial numbers in the organs of the immunized and challenged mice are reduced 10- to 100-fold compared with those in unimmunized mice. Prior studies have shown that BCG immunization of $\beta_2m^{-/-}$ mice (which lack MHC class I molecules and, consequently, are deficient in CD8 T cells) (29) or TNF-p55 $receptor^{-/-}$ mice (13) increased survival time following *M. tuberculosis* challenge, although the mice still succumbed to the infection. In contrast, BCG immunization of MHC class $II^{-/-}$ mice for 3 mo did not increase mean survival time following virulent *M. tuberculosis* challenge (Table I). Curiously, no adverse effects of BCG infection were observed despite the absence of CD4 T cells in these mice. As an alternative immunization strategy, in case BCG immunization was not adequate to induce protective CD8 T cell responses, MHC class $II^{-/-}$ mice were immunized with virulent *M. tuberculosis* (1×10^3 i.v.) or BCG (1×10^5 i.v.) for 4 wk, then treated with the antibiotic isoniazid for 4 wk. Ten days after cessation of antibiotic treatment, mice were challenged with virulent *M. tuberculosis* (5×10^5 i.v.). Bacterial numbers in lungs, liver, and spleen of BCG- or *M. tuberculosis*-immunized mice were 100- to 1000-fold higher than those in wild-type immunized mice at 3 and 6 wk postinfection and were similar to those in unimmunized MHC class $II^{-/-}$ mice (data not shown). The mean survival time of these mice was also unchanged compared with that of unimmunized control mice (Table I); there was no difference in protection between mice immunized with BCG and those immunized with *M. tuberculosis* ($p = 0.61$). Thus, immunization was not effective in protecting against subsequent virulent *M. tuberculosis* challenge in the absence of MHC class II Ag presentation.

Granuloma formation in the absence of CD4 T cells

In immunocompetent mice, the response to *M. tuberculosis* includes the formation and maintenance of granulomas, composed of CD4 and CD8 T cells surrounding epithelioid macrophages (29). Granuloma formation was assessed in tissue sections from wild-type, MHC class $II^{-/-}$, and $CD4^{-/-}$ mice at various times postinfection. At 1 wk postinfection liver sections from $CD4^{-/-}$ and MHC class $II^{-/-}$ mice contained only 20–25% as many granulomas as liver tissue from wild-type control mice (Table II). However, by 2 wk postinfection the numbers of granulomas in liver sections from the wild-type and CD4-deficient mice were equivalent and remained so over the course of infection (Table II). A similar pattern was observed in the lungs (data not shown).

In general, the liver and lung granulomas in the CD4 T cell-deficient mouse strains were more disorganized than those in wild-type mice and were more likely to be comprised of uniform histiocytic or mixed histiocytic and granulocytic cells (Fig. 3, *A–C*).

Table II. *Granuloma formation in the presence and absence of CD4 T cells*[a]

	Wild Type		MHC Class $II^{-/-}$		$CD4^{-/-}$	
Days Postinfection	No./10× field	% $NOS2^{+}$	No./10× field	% $NOS2^{+}$	No./10× field	% $NOS2^{+}$
7	4.3	41	0.9*	38	1.2*	28*
14	9.3	92	8.4	63**	10.9	61**
21	5.7	71	5.9	69	ND	ND
35	7.5	82	12.4	ND	9.6	57**

[a] Liver sections from mice at various times postinfection were stained by hematoxylin and eosin and microscopically examined; the number of granulomas per 10× field were recorded. Sections from three to four mice per group and per time point were assessed; five to eight 10× fields for each slide were counted. Mean number of granulomas per 10× field are reported. Serial sections were stained with anti-NOS2 Ab, and the percentage of positively staining granulomas was determined. Two to four samples from each group at each time point were examined, and results were combined to obtain the overall percentage of granulomas staining positively for NOS2.

ND, not done.

* $p < 0.001$ compared with wild type.

** $p < 0.05$ compared with wild type.

Organized granulomas consisting of an outer rim of mononuclear cells and central aggregates of large, epithelioid histiocytes were more commonly seen in wild-type animals. Well-organized granulomas were, however, also present in the mutant mice. Lung tissue from wild-type, $CD4^{-/-}$, and MHC class $II^{-/-}$ mice were evaluated and scored histopathologically for the imflammatory processes present. These included the infiltration of mixed, but primarily lymphohistiocytic, cells throughout interstitial regions (interstitial pneumonitis), the presence of circumscribed foci of granulomatous inflammation, inflammatory cell infiltration into alveolar air spaces (alveolitis), and histomorphological evidence of necrosis (Fig. 3, *D–F*). Generally, wild-type mice demonstrated less severe interstitial pneumonitis, necrosis, and alveolitis than their transgenic counterparts, especially in the later stages of infection. Consistent, significant differences between the histology of $CD4^{-/-}$ and MHC class $II^{-/-}$ tissues were not recognized within these scoring categories.

Immunohistochemistry was performed to determine the extent of NOS2 staining within granulomas in the control and CD4-deficient mice. Usually NOS2 production occurs in activated macrophages, although it can also increase due to bacterial burden. At 2 wk postinfection, the numbers of granulomas in liver sections staining positively for NOS2 was lower in the MHC class $II^{-/-}$ and $CD4^{-/-}$ mice compared with control mice (Table II). By 3 wk postinfection the percentage of liver granulomas staining positively for NOS2 protein was similar among wild-type and CD4-deficient mice (Table II). NOS2 staining in lung followed a similar pattern (data not shown). Thus, expression of NOS2 by macrophages was delayed, but not absent, in the CD4 T cell-deficient mouse strains. The early production of RNI by macrophages may be essential to control of the infection.

T cell subsets

It was previously reported that $CD4^{-/-}$ mice had significant numbers of double-negative T cells ($CD4^{-}8^{-}$), and that such cells were capable of proliferating and producing IFN-γ in an MHC class II-restricted manner in response to leishmanial Ag (30). This phenomenon was not observed in MHC class $II^{-/-}$ mice. In those studies $CD4^{-/-}$ mice were resistant to *Leishmania major* challenge, while MHC class $II^{-/-}$ mice were susceptible. In our acute tuberculosis model, $CD4^{-/-}$ mice had a longer mean survival time than MHC class $II^{-/-}$ mice, although both strains of mice were much more susceptible than wild-type littermate controls (Fig. 1). Therefore, we examined the presence and function of the double-negative T cell population in $CD4^{-/-}$ and MHC class $II^{-/-}$ mice following infection with *M. tuberculosis*. The T cell phenotype of spleen cells at 0, 14, and 24 days postinfection was assessed by Ab

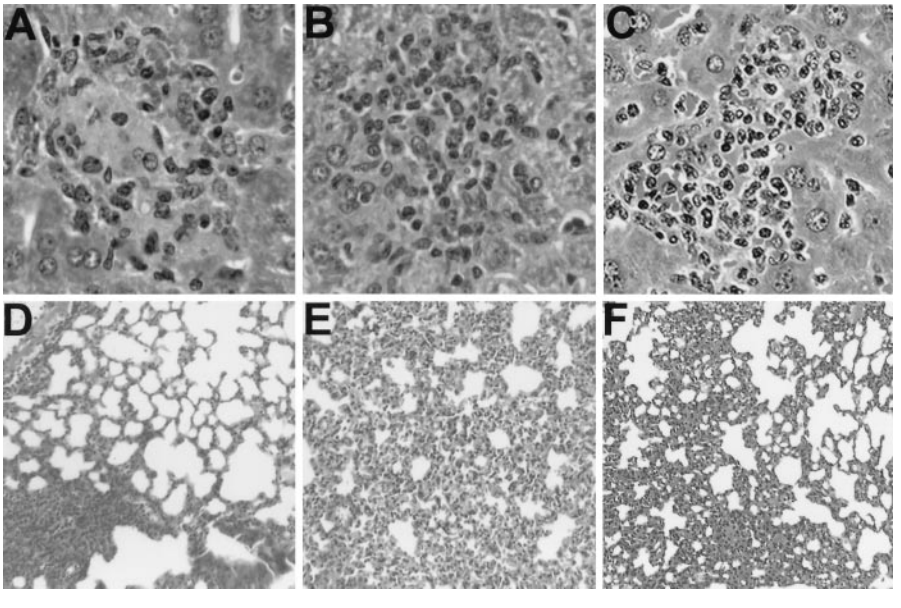

FIGURE 3. Tissue pathology in infected mice. Wild-type (*A* and *D*), MHC class $II^{-/-}$ (*B* and *E*), and $CD4^{-/-}$ (*C* and *F*) mice were infected with *M. tuberculosis*, and tissues were harvested at 2 wk postinfection. Liver (*A–C*) and lung (*D–F*) sections were stained by hematoxylin and eosin.

Table III. *T cell subsets following infection of wild-type and CD4-deficient mice*[a]

Mouse Strain	Days Postinfection	% $CD4^+$	% $CD8^+$	% $CD4^-8^-$
Wild type	0	58	27	9
MHC class $II^{-/-}$	0	8	65	23
$CD4^{-/-}$	0	1	67	31
Wild type	14	68	22	10
MHC class $II^{-/-}$	14	6	69	23
$CD4^{-/-}$	14	1	68	31
Wild type	24	62	19	15
MHC class $II^{-/-}$	24	14	50	33
$CD4^{-/-}$	24	4	60	34

[a] Spleen cells were obtained from mice infected with *M. tuberculosis* and triple-stained for cell surface expression of CD3, CD4, and CD8. Cells were analyzed by flow cytometry. $CD3^+$ cells were gated and analyzed for CD4 and CD8 expression. The percentages of $CD3^+$ cells that are also positive for either CD4 or CD8, or neither, are shown. The average values for two to four mice per group per time point are reported.

staining of cell surface markers and flow cytometry. *M. tuberculosis* infection caused a slight increase in double-negative $CD3^+$ cells in wild-type mice (Table III). As described previously (26, 27), MHC class $II^{-/-}$ and $CD4^{-/-}$ mice had higher numbers of CD8 T cells compared with wild-type mice due to the deficiency in CD4 T cells. Compared with wild-type mice, a much higher proportion of spleen cells from both MHC class $II^{-/-}$ and $CD4^{-/-}$ mice was $CD3^+$ $CD4^-8^-$ cells even before infection (Table III), and little increase in the percentage of $CD4^-8^-$ T cells was observed following infection. In contrast to the reported findings in the *L. major* system, we did not observe substantial differences between the two CD4 T cell-deficient mouse strains in the numbers of double-negative T cells following *M. tuberculosis* infection. Thus, the increased survival of $CD4^{-/-}$ mice compared with that of MHC class $II^{-/-}$ mice does not appear to be due to an increase in the number of double-negative T cells.

In vitro T cell proliferation

The ability of T cells from the CD4 T cell-deficient mice to proliferate in response to mycobacterial Ags was examined by stimulating spleen cells from mice at 2 and 4 wk post-*M. tuberculosis* infection with Con A or PPD for 3 days and measuring the incorporation of [^{3}H]thymidine. Although T cells from uninfected control (littermate) mice responded strongly to Con A, T cells from infected wild-type mice had lower responses, suggesting a suppressive effect of *M. tuberculosis* infection, which has been described previously (31, 32). Spleen cells from infected wild-type mice showed proliferation in response to PPD (Fig. 4*A*), although the stimulation index was low. In contrast, there was little or no proliferation of spleen cells from MHC class $II^{-/-}$ or $CD4^{-/-}$ mice in response to PPD. Con A-induced proliferation was lower

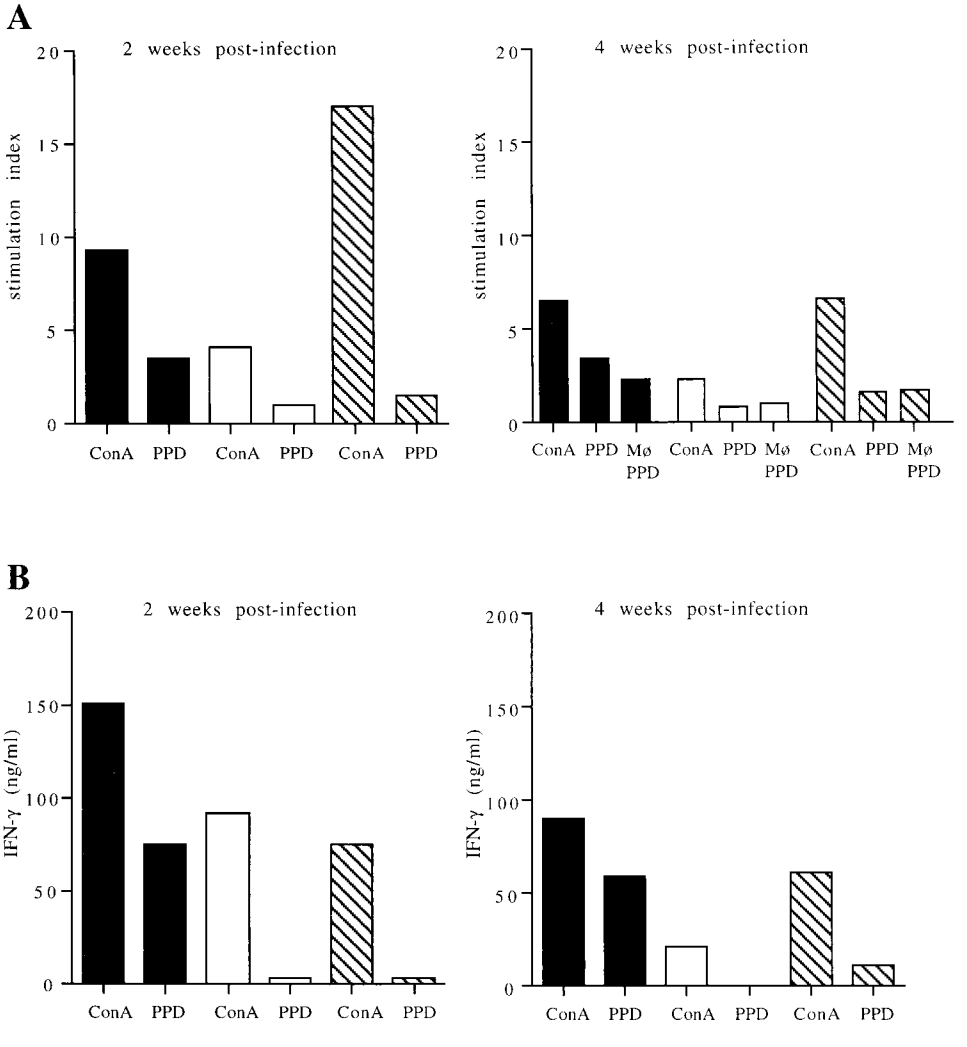

FIGURE 4. T cell proliferation and IFN-γ production by wild-type and mutant mice. Wild-type (filled bars), MHC class $II^{-/-}$ (open bars), and $CD4^{-/-}$ mice (crosshatched bars) were infected with 2×10^5 *M. tuberculosis* bacilli, i.v. At 2 and 4 wk postinfection spleen cells were stimulated with medium alone, Con A, PPD, or wild-type macrophages and PPD. *A*, Proliferation was measured by [^{3}H]thymidine incorporation after 3 days of culture and is represented as the stimulation index compared with medium for each group. *B*, IFN-γ was measured by sandwich ELISA in the cultures described above. Two mice per group were used, and the experiment was repeated twice.

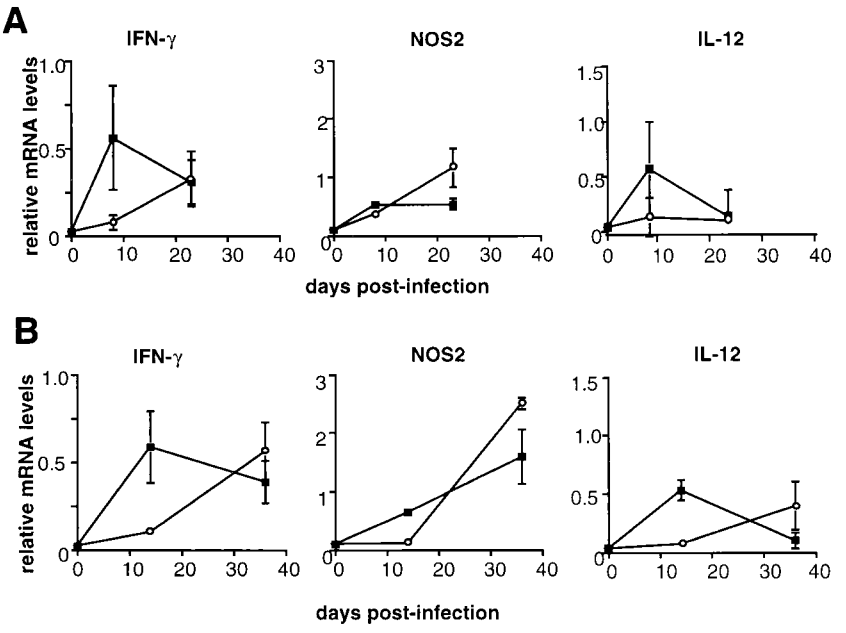

FIGURE 5. mRNA expression in lungs of infected mice. *A*, MHC class II (○) and wild-type (■) mice. *B*, CD4$^{-/-}$ (○) and wild-type (■) mice. Mice were infected with 5×10^5 *M. tuberculosis* bacilli i.v. RNA from lung tissue was reverse transcribed and standardized for HPRT expression using RT-PCR and a competitor plasmid. RT-PCRs with competitor plasmid were performed with primers specific for IFN-γ, NOS2, and IL-12. PCR products on agarose gels were stained with Sybr Green and quantitated by PhosphorImager. Data are reported as relative mRNA levels, using the HPRT ratio as a correction factor for each sample. Thus, a relative mRNA level of 1 is equivalent to the HPRT mRNA level. Day 0 indicates uninfected mice. Three or four mice per time point were analyzed, and the mean is represented here with the SE. All PCRs were performed at least three times.

in MHC class II$^{-/-}$ spleen cells, but was similar to that in wild-type in CD4$^{-/-}$ spleen cells (Fig. 4*A*). Double-negative T cells present in the spleen cells of the mutant strains did not appear to proliferate in response to MHC class II presentation of Ag. Addition of wild-type macrophages (i.e., those with MHC class II molecules) did not result in increased proliferation to PPD by spleen cells from either CD4 T cell-deficient mouse strain (Fig. 4*A*). At later time points in the infection, increased stimulation of the wild-type spleen cell response to PPD was observed (our unpublished observations), but the limited life span of infected CD4 T cell-deficient mice precluded comparisons of T cell stimulation at the later time points.

In vitro cytokine production by splenocytes from M. tuberculosis*-infected mice*

Alteration in cytokine production in the absence of CD4 T cells was an obvious possibility to explain the inability of the MHC class II$^{-/-}$ and CD4$^{-/-}$ mice to control infection. We initially examined IFN-γ production by T cells in response to PPD in vitro using ELISA. Spleen cells from infected wild-type mice produced IFN-γ in response to PPD as well as to the nonspecific stimulus of Con A (Fig. 4*B*). In contrast, IFN-γ production in response to PPD was very low or undetectable in spleen cell cultures from MHC class II$^{-/-}$ or CD4$^{-/-}$ mice, although Con A induced IFN-γ in these cultures (Fig. 4*B*). Addition of wild-type macrophages (i.e., those with MHC class II molecules) as APCs to the cultures in the presence of PPD did not increase the production of IFN-γ (data not shown).

Cytokine mRNA expression in vivo following M. tuberculosis *infection*

In vitro proliferation assays may not be indicative of the actual cytokine production by T cells in vivo, especially over the course of infection, since it is a restimulation assay. In addition, this assay primarily measures MHC class II-restricted proliferation, and the contribution of MHC class I-restricted T cells (CD8$^+$ or CD4$^-$8$^-$) is not well represented. We were also interested in the cytokine response in the lungs; bulk T cells from lungs are difficult to obtain in sufficient numbers for in vitro proliferation assays. To assess in vivo cytokine production, we used QC-RT-PCR (28) to compare gene expression in the tissues of control and knockout mice at various times postinfection. In the lungs of MHC class II$^{-/-}$ mice, IFN-γ expression was approximately 5-fold lower than that in wild-type lungs early in infection, but expression levels were similar between the two groups at 3 wk postinfection (Fig. 5*A*). Expression of NOS2 was similar between the mice in the lungs (Fig. 5*A*). IL-12 expression was also decreased in the lungs of MHC class II$^{-/-}$ mice at 1 wk postinfection, but IL-12 expression in the control mice decreased by 3 wk postinfection to the level seen in the MHC class II$^{-/-}$ mice (Fig. 5*A*). IL-10 expression in the lungs of all mice was low, and IL-4 mRNA was not detected at any time point (data not shown). Using our RT-PCR assay, we were unable to detect IL-2 mRNA in the lungs or spleens of either wild-type or CD4-deficient mice, whether uninfected or infected (data not shown).

Gene expression in the CD4$^{-/-}$ mice appeared to correspond to that observed in MHC class II$^{-/-}$ mice, in that IFN-γ expression was reduced approximately 5-fold compared with that in wild-type controls early in infection, but returned to wild-type levels as the infection progressed (Fig. 5*B*). There was a more obvious decrease in NOS2 expression early in infection in the CD4$^{-/-}$ mice compared with wild-type controls, but it also returned to wild-type levels later in infection (Fig. 5*B*). IL-12 expression was reduced in CD4$^{-/-}$ mice at 2 wk postinfection compared with that in wild-type mice (Fig. 5*B*). IL-4 and IL-2 mRNA were undetectable at all time points (data not shown).

In vivo intracellular cytokine staining

The data obtained by RT-PCR suggested that IFN-γ expression in the lungs was lower in the absence of CD4 T cells at early time points after infection, but quickly returned to wild-type levels as the infection progressed. Using QC-RT-PCR on whole tissue RNA, it was only possible to examine overall IFN-γ production, and no information regarding the cells responsible for IFN-γ production could be gathered. There was also variability among mice using the QC-RT-PCR assay. For these reasons, we used intracellular cytokine staining of lung and spleen cells directly from the infected mice to assess the potential for each cell type to produce cytokines in vivo in response to *M. tuberculosis* infection.

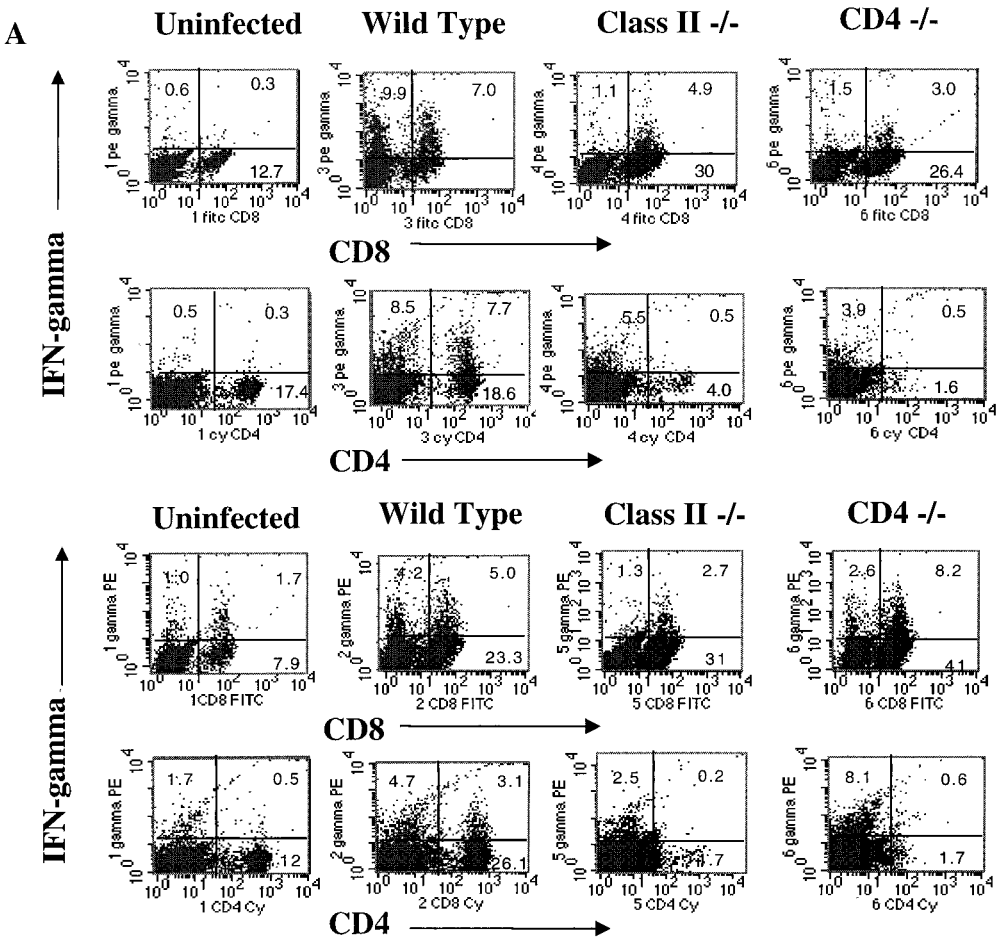

FIGURE 6. Intracellular cytokine staining of lung cells from infected mice. Lung cells were obtained from uninfected mice or from wild-type, MHC class $II^{-/-}$, and $CD4^{-/-}$ mice at various times postinfection. Cells were stimulated for 6 h with anti-CD3 and anti-CD28 Abs in the presence of monensin, stained for CD4 and CD8, fixed in paraformaldehyde, permeabilized, and stained for intracellular IFN-γ. Cells were gated on lymphocytes by size and analyzed by three-color flow cytometry. A representative mouse of each group at 2 and 6 wk postinfection is shown here, with the data from multiple mice and time points represented in Fig. 7.

Lung and spleen cells from infected mice were obtained, stimulated with anti-CD3 and anti-CD28 Abs, treated with monensin for only 6 h, stained for cell surface molecules, permeablized, and stained for intracellular cytokine protein. Lung and spleen cells from uninfected mice were used as controls; these cells produced little or no IFN-γ or IL-4 after stimulation as described above (Fig. 6), indicating that only in vivo activated T cells will produce cytokines upon short term (4–6 h) stimulation with anti-CD3 and anti-CD28 Abs. Based on these results, we chose this technique to compare cytokine production by subsets of T cells without in vitro culture and obtain a clearer picture of cytokine production in vivo during an infection. A representative experiment is shown in Fig. 6, while Fig. 7 shows the combined results from at least three experiments over the course of infection.

In wild-type mice, numbers of T cells, both $CD4^+$ and $CD8^+$, increased in the lungs following infection (our unpublished observations). In the mutant mice an increase in CD4 T cells was not observed, since these mice are lacking CD4 T cells. However, CD8 T cells in the lungs were increased substantially in both MHC class $II^{-/-}$ and $CD4^{-/-}$ mice following infection (data not shown).

Lung cells were used to analyze IFN-γ production following infection, since the lung is the most relevant site of bacterial infection in tuberculosis. In wild-type mice at 1 wk postinfection, IFN-γ production by lung cells was very low (Fig. 7). By 2 wk postinfection, IFN-γ production was increased, with approximately 11% of the lung lymphocytes (gated by size) producing IFN-γ (Figs. 6 and 7). IFN-γ was produced by both CD4 and CD8 T cells, with higher production by the CD4 T cell subset. By 4 and 6 wk postinfection, IFN-γ production had stabilized (~10% of lymphocytes), with contributions from both CD4 and CD8 T cells (Figs. 6 and 7). IL-4 was not detected in lung cells at any time point (data not shown).

IFN-γ production in the lungs of MHC class $II^{-/-}$ and $CD4^{-/-}$ at various times postinfection was compared with that in wild-type mice (Figs. 6 and 7). At 7 days postinfection, IFN-γ production was low in all groups. At 14 days postinfection, IFN-γ production in MHC class $II^{-/-}$ and $CD4^{-/-}$ mice was only 44% of that in wild-type mice ($p < 0.01$). At 4 wk postinfection, total IFN-γ levels in the lungs of wild-type and CD4-deficient mice were similar. In both MHC class $II^{-/-}$ and $CD4^{-/-}$ lungs, CD8 T cells were responsible for the majority of IFN-γ production, with $CD4^-8^-$ cells accounting for only a small proportion of IFN-γ (Fig. 6). The apparent decrease in total IFN-γ production in the MHC class $II^{-/-}$ lung cells at the 6 wk point was probably due to the presence of necrosis and cells in poor condition in the lungs, since the mean survival time is approximately 6 wk. IL-4 was not detectable at any time point in the wild-type or CD4-deficient mice (data not shown).

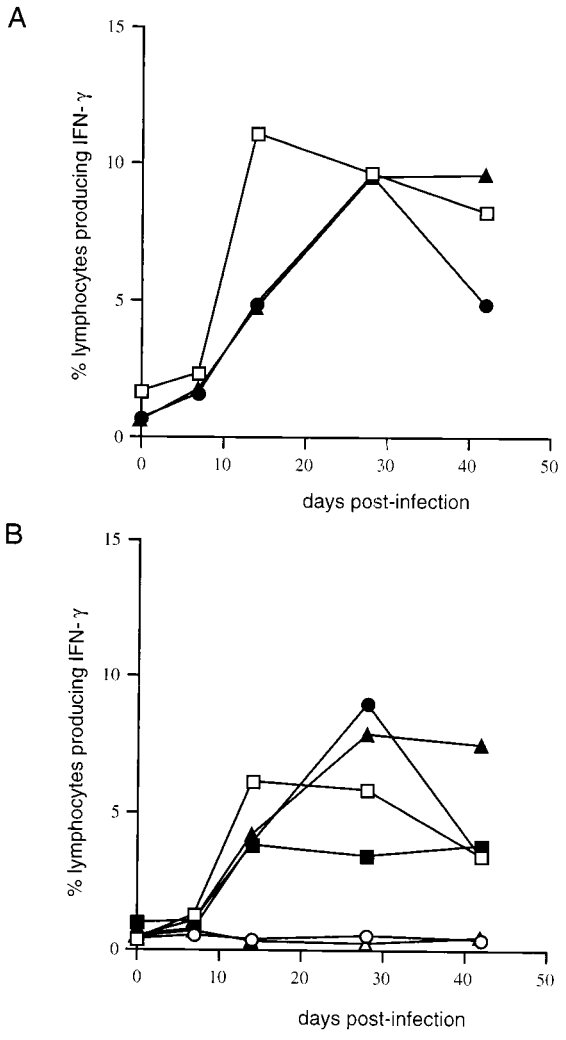

FIGURE 7. Intracellular cytokine staining of lung cells from infected mice. Lung cells from mice at 0, 1, 2, 4, and 6 wk postinfection were analyzed by intracellular cytokine staining as described in Fig. 6. The combined results of three to eight mice per time point are shown here. *A*, Total percentage of gated lymphocytes staining positively for IFN-γ in wild-type (□), MHC class $II^{-/-}$ (●), and $CD4^{-/-}$ (▲). *B*, Percentage of gated lymphocytes that are $CD4^+$ IFN-γ^+ (open symbols) or $CD8^+$ IFN-γ^+ (filled symbols) in lungs of wild-type (squares), MHC class $II^{-/-}$ (circles), or $CD4^{-/-}$ (triangles) mice.

Spleen cells from these mice gave similar results as lung cells in intracellular staining experiments, but the overall percentage of IFN-γ-producing T cells observed was lower (data not shown). This is probably due to the large number of resident, nonspecific T cells in the spleen. In the lung, 2- to 5-fold more T cells were isolated from infected mice compared with uninfected mice (our unpublished observations), suggesting that specific T cells migrate to or proliferate at the site of infection in the lung. Uninfected mice have a relatively small number of T cells. Thus, a higher percentage of the T cells found in the lungs are likely to be mycobacterium-specific IFN-γ-producing T cells.

Discussion

CD4 T cells have long been believed to be important in protection against tuberculosis. The loss of CD4 T cells during HIV infection has been accepted as a trigger for reactivation of latent tuberculosis as well as a factor responsible for the increased susceptibility of HIV^+ persons to infection with *M. tuberculosis*. In murine models the production of IFN-γ is essential to limiting replication of *M. tuberculosis* organisms (11, 12). This cytokine is also likely to be important in human tuberculosis, as humans deficient in receptors for IFN-γ or IL-12 are very susceptible to mycobacterial infections (33–36). Numerous studies report that CD4 T cells from *M. tuberculosis*-infected mice or humans proliferate in response to mycobacterial Ags and produce IFN-γ. An obvious conclusion from the previous studies was that the primary role of CD4 T cells in protection against tuberculosis is the production of IFN-γ. Here, we addressed how a loss of CD4 T cells affected the ability of mice to control tuberculosis. Mice deficient in CD4 T cells were strikingly more susceptible to *M. tuberculosis* infection than were control mice. Surprisingly, the level of IFN-γ production in the lungs was only transiently decreased early in infection compared with that in wild-type mice. These data suggest that early production of IFN-γ by CD4 T cells is essential to control of this infection, and that IFN-γ production by other cells cannot substitute for the CD4 T cell contribution. Our data do not rule out an IFN-γ-independent function of CD4 T cells in controlling tuberculosis. In fact, it is likely that the immunodeficiency in HIV^+ patients that enhances susceptibility to tuberculosis is complex and not simply due to decreased IFN-γ production by CD4 T cells.

MHC class $II^{-/-}$ and $CD4^{-/-}$ mice infected with *M. tuberculosis* had much higher bacterial numbers in the organs compared with control mice and succumbed to the infection. MHC class $II^{-/-}$ mice were shown previously to be susceptible to *M. tuberculosis* infection, but a transient plateau of CFU in the lung and spleens 20–60 days postinfection was reported, which was interpreted as the contribution of CD8 T cells to protection in this model (25). We did not observe such a plateau; however, bacterial numbers were calculated differently in our study: CFU per gram tissue were reported by Tascon et al., while we calculated the total number of CFU per organ. This may account for the apparent discrepancy in results, as the organs of MHC class $II^{-/-}$ and $CD4^{-/-}$ mice increase in size relative to those in wild-type mice as the infection progresses.

Although MHC class $II^{-/-}$ and $CD4^{-/-}$ mice were more resistant than IFN-γ-deficient (11, 12) or TNF-α-deficient (13) mice, the mean survival time was similar to that in β_2m-deficient mice (29). It is simplistic to compare gene-disrupted mice to obtain a meaningful picture of the relative protective effects of each immune system component. However, it is clear that both CD4 and CD8 T cells are important in protection against tuberculosis, and each subset is incapable of substituting for the other in providing this protection. Unlike mice deficient in MHC class I molecules (29), prior immunization with BCG or *M. tuberculosis* did not extend the mean survival time of the MHC class $II^{-/-}$ mice. These results demonstrate that immunization depends on MHC class II Ag presentation and confirm the central role of MHC class II molecules and CD4 T cells in controlling tuberculosis. The data available to date strongly indicate that CD4 and CD8 T cells are performing at least some different functions during infection, and studies to define these functions are essential to our understanding of this disease and the most effective means for vaccination.

Spleen cells from *M. tuberculosis*-infected MHC class $II^{-/-}$ and $CD4^{-/-}$ mice were impaired in the ability to proliferate and produce IFN-γ in response to PPD in vitro. However, in vitro proliferation assays under the conditions used here favor the proliferation of CD4 T cells, rather than CD8 T cells, and may give an inaccurate picture of the in vivo potential of the T cells to produce

IFN-γ. Thus, we turned to assays that would allow assessment of in vivo production of IFN-γ.

IFN-γ mRNA levels in the lungs of CD4 T cell-deficient mice were approximately 5-fold lower than those in wild-type mice early in infection. However, by 3 wk postinfection there were no discernible differences in levels of IFN-γ mRNA among CD4 T cell-deficient and wild-type mice. IL-12 p40 mRNA, which is enhanced by IFN-γ production, was also deficient only at early time points after infection. Intracellular cytokine staining of lung cells directly isolated from infected mice was used to assess the potential for various cell types to produce IFN-γ during infection. This assay identifies cells capable of IFN-γ production, but may overestimate the percentage of cells actually producing IFN-γ at any one time point during the infection. The results obtained were similar to those obtained with RT-PCR in terms of kinetics of IFN-γ production in the lungs. At 2 wk postinfection mice deficient in CD4 T cells produced only 44% of the IFN-γ produced by wild-type mice. While IFN-γ production by CD8 T cells was approximately the same in CD4 T cell-deficient and wild-type mice at this time point, the contribution of the CD4 T cells to IFN-γ was obviously absent in the mutant mice. This suggests that IFN-γ production at the early time points of infection must come from CD4 T cells to be effective, and that CD8 T cell IFN-γ production is insufficient to control the infection. Interestingly, by 4 wk postinfection, IFN-γ production by the CD8 T cell subset in the CD4 T cell-deficient mice increased, so that total IFN-γ production was similar to that in wild-type mice (Fig. 7), consistent with mRNA data. However, this IFN-γ production was apparently insufficient to control the infection in the absence of CD4 T cells.

$CD4^{-/-}$ mice had a longer mean survival time than MHC class $II^{-/-}$ mice following *M. tuberculosis* infection. A discrepancy in susceptibility between these two mouse strains was also reported following *L. major* infection, in which the protection of $CD4^{-/-}$ mice was attributed to MHC class II-restricted IFN-γ production by *Leishmania*-specific $CD4^{-}8^{-}$ T cells (30). No difference in the numbers of $CD4^{-}8^{-}$ cells between MHC class $II^{-/-}$ and $CD4^{-/-}$ mice following *M. tuberculosis* infection was observed, nor did these cells proliferate or produce IFN-γ in response to mycobacterial Ag. In vivo, these cells did not appear to contribute to IFN-γ production, since most IFN-γ intracellular staining following stimulation of lung cells was contributed by CD8 T cells in the mutant mice. The reasons for the difference in susceptibility to tuberculosis between these two strains is unclear, but appears not to be related to the direct contribution of $CD4^{-}8^{-}$ T cells.

Early IFN-γ production may not be the only role for CD4 T cells in protection against tuberculosis. The effect of CD4 T cells on CD8 T cell development and function must be taken into account, since CD8 T cells are important in the control of murine tuberculosis (29, 37). The number of CD8 T cells increases in the lungs following *M. tuberculosis* infection in the presence or the absence of CD4 T cells (our unpublished observations), and these cells are capable of producing IFN-γ. However, another role for CD8 T cells in protection against tuberculosis may be as CTL. The absence of CD4 T cells in the mutant mice may prevent the CD8 T cells from becoming CTL, which may contribute to the susceptibility of the mutant mice to tuberculosis. However, effective CTL responses during viral infections have been demonstrated in MHC class $II^{-/-}$ and $CD4^{-/-}$ mice (27, 38–40). In vitro, the requirement for CD4 T cells in the generation of CD8 CTL can be bypassed by modulation of the APC by either ligation of CD40 or viral infection (41–43). This suggests that in vivo, infection with a pathogen such as *M. tuberculosis* may also bypass the need for CD4 T cells in eliciting CD8 T cell responses. Certainly, the data presented here indicate that CD4 T cells are not necessary for eliciting CD8 T cells that produce IFN-γ during mycobacterial infection; studies are currently underway to assess the presence of mycobacteria-specific CTL in the infected mutant mice.

Another role for CD4 T cells in eliciting CD8 T cells is the production of IL-2. We were unable to detect IL-2 transcripts in the lungs or spleens of wild-type or mutant infected mice; this may be due to the time points chosen for analysis. However, the double-negative T cells ($CD4^{-}8^{-}$) in the mutant mice or even the CD8 T cells may be a source of sufficient IL-2 to drive T cell expansion. Previous studies have suggested that such cells may produce IL-2 in the absence of CD4 T cells (41).

Granulomas are believed to prevent the spread of infection throughout the organs. Both CD4 and CD8 T cells are present in the granulomas of wild-type mice, suggesting that each cell type contributes to the maintenance and function of the granuloma (29). Granuloma formation was delayed for about 1 wk in the CD4 T cell-deficient mice, suggesting that CD4 T cells are important in the initial formation of the granuloma and also for the elimination of bacilli within the granuloma. Although the numbers of granulomas present in the livers and lungs of CD4-deficient mice reached wild-type levels by 2 wk postinfection, the bacterial burden in these mice was not brought under control, indicating a loss of granuloma function. Indeed, in general, the granulomas present in the CD4 T cell-deficient mice were less organized than those in the tissues of wild-type mice.

NOS2 expression is dependent on IFN-γ. A delay in NOS2 gene and protein expression was observed in the CD4 T cell-deficient mice compared with that in control mice. By 3 wk postinfection, NOS2 protein levels were similar among CD4 T cell-deficient and control mice, yet the mutant mice were unable to control the infection. As we observed in TNF receptor-deficient mice, later production of RNI is insufficient to control an ongoing infection. These data lend support to the idea that the early expression of antimycobacterial mechanisms of macrophages is crucial to the control of tuberculosis.

In summary, we have shown that CD4 T cells are required for resistance to tuberculosis, and that the absence of CD4 T cells results in a delay in IFN-γ production as well as a delay in NOS2 expression and granuloma formation. Thus, early production of IFN-γ is likely to be a major role for CD4 T cells in protection against tuberculosis. The contribution of CD8 T cells to IFN-γ production is obvious after 2 wk of infection, but the CD8 T cell subset is insufficient to compensate for the loss of CD4 T cells. It is clear that the early immunologic events in *M. tuberculosis* have profound consequences for the outcome of infection and disease. These findings have important implications for tuberculosis and AIDS, and the murine models described here may be useful to study the effects of tuberculosis on CD4 T cell-deficient subjects as well as immunotherapies and drug regimens that may be useful in an immunocompromised individual.

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