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Contribution of α/β and γ/δ T lymphocytes to immunity against *Mycobacterium bovis* Bacillus Calmette Guérin: studies with T cell receptor-deficient mutant mice*

Mutant mice with defined T cell deficiencies were infected with *Mycobacterium bovis* bacillus Calmette Guérin (BCG) and the relative contribution of α/β T cells and γ/δ T cells to the host immune response was assessed. Recombinase activating gene (RAG-1)$^{-/-}$ mutants as well as T cell receptor (TcR) β$^{-/-}$, but not TcR-δ$^{-/-}$, mutants succumbed to *M. bovis* BCG infection and failed to develop granulomatous lesions. Antigen-induced IFN-γ production by spleen cells *in vitro* was abrogated in RAG-1$^{-/-}$ mutants and markedly diminished in TcR-β$^{-/-}$ and TcR-δ$^{-/-}$ mice. Reconstitution experiments suggest that both α/β and γ/δ T cells are essential for antigen-specific IFN-γ secretion. Our data formally prove the crucial role of α/β T cells and reveal accessory functions of γ/δ T cells in optimum immunity against *M. bovis* BCG.

1 Introduction

Intracellular bacteria are microbial pathogens which share several common features [1, 2]. They preferentially inhabit resting macrophages which provide a protective niche within the host. Acquired resistance is mediated by T lymphocytes, and antibodies play only a minor, if any, role. T lymphocytes secrete macrophage-activating cytokines, in particular IFN-γ, which activate antibacterial mechanisms in infected macrophages [3–6]. The activated macrophage is the major effector cell of acquired host resistance against intracellular bacteria. While granulomatous lesions at the site of microbial growth, promote microbial containment to distinct foci, thus contributing to protection, they also cause tissue destruction, thus participating in pathogenesis [7]. Upon local injection of soluble antigens, diffuse and transient reactions develop. Although these so-called delayed-type hypersensitivity (DTH) reactions are mediated by T cells, their correlation with protective immunity remains unclear [8].

Intracellular bacteria include a variety of pathogens, such as *Listeria monocytogenes, Salmonella typhimurium* and the pathogenic mycobacteria, *Mycobacterium tuberculosis, Mycobacterium bovis* and *Mycobacterium leprae*. In spite of their common intracellular habitat, these microbes prossess distinct characteristics. For example, *L. monocytogenes*, which is widely used for experimental analyses of the mechanisms responsible for acquired protection against intracellular pathogens, is an acute infection which is readily overcome once T lymphocytes had been fully activated [2]. In contrast, mycobacterial pathogens such as *M. tuberculosis* and *M. bovis*, the etiologic agents of tuberculosis, persist for long periods of time, and the microbe is generally not fully eradicated even after activation of specific T lymphocytes [1]. Rather, T cell-mediated immunity induces mycobacterial growth control within distinct foci. Even the vaccine strain *M. bovis* BCG persists in the presence of potent T cell-mediated immunity, apparently without causing major harm to the host [9].

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Abbreviations: p.i.: Post infection **RAG:** Recombinase activating gene

Key words: T cell receptor-deficient mice / BCG infection / Cytokine production

On the basis of their antigen-specific receptors, T lymphocytes segregate into two major populations [10]. The vast majority of peripheral T cells in man and mouse expresses a TcR composed of an α and a β chain, whereas a minor population of peripheral T cells uses the γ- and δ-chain combination. While the central role of T lymphocytes in immunity to mycobacteria is without doubt, little is known about the relative contribution of α/β T cells and γ/δ T cells to protection and pathogenesis [1]. T cell-dependent antibacterial functions have generally been ascribed to α/β T lymphocytes, primarily because of their abundance and due to our extensive knowledge of this T cell population. Circumstantial evidence suggests a particular role of γ/δ T cells in immunity to mycobacteria [11–14]. γ/δ T cells have been found to accumulate in granulomatous lesions in patients at certain stages of mycobacterial infection [13, 14]. In the murine system, γ/δ T lymphocytes are activated after immunization with mycobacteria and accumulate early at the site of mycobacterial growth [11, 12]. Therefore, there is a demand for a more precise definition of the respective impact of α/β T cells and γ/δ T cells on host defence against chronic mycobacterial infections.

Recently, mutant mice have been established by homologous recombination with defined deficiencies in the T cell system [15]. These include RAG-1$^{-/-}$ mice which are devoid of all mature B and T lymphocytes; TcR-β$^{-/-}$ mice without α/β T cells, but virtually normal levels of γ/δ T cells, and TcR-δ$^{-/-}$ mice with virtually normal α/β T cells, but without γ/δ T cells [16–18]. We have taken advantage of these gene-disruption mutants to assess the relative contribution of α/β T cells and γ/δ T cells to the

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immune response to the attenuated anti-tuberculosis vaccine strain *M. bovis* BCG. Our data formally prove the central role of α/β T cells in major aspects of the immune response *in vivo* and *in vitro*, *i.e.* microbial growth control in infected organs, formation of granulomatous lesions at the site of mycobacterial replication, development of DTH reactions in response to challenge with purified protein derivative (PPD), and antigen-induced IFN-γ production. Our data also reveal an auxiliary function of γ/δ T cells in anti-mycobacterial immunity: in TcR-$\delta^{-/-}$ mutants, growth of *M. bovis* BCG was modestly, but still demonstrably, increased, and antigen-induced IFN-γ secretion *in vitro* was markedly diminished. Cell reconstitution experiments suggest that IFN-γ production in response to mycobacterial antigens depends on interactions between α/β T cells and γ/δ T cells. Hence, although α/β T cells are essential for acquisition of cell-mediated immunity and γ/δ T cells are insufficient by themselves, our data reveal a significant assistance of γ/δ T cells in immunity to *M. bovis* BCG.

2 Materials and methods

2.1 Mice

The TcR-$\beta^{-/-}$, TcR-$\delta^{-/-}$ and RAG-$1^{-/-}$ mutant mice created by gene targeting techniques have been described previously [16–18]. Homozygous mutants and their respective heterozygous littermates were backcrossed to C57BL/6 mice. Mice used in this study were from the third or later backcrosses. The homozygous mutant animals and their heterozygous littermates were bred and maintained in the animal facility of the University of Ulm under controlled specific pathogen-free conditions and were employed in experiments at 7–8 weeks of age. In a given experiment, animals were age and sex matched.

2.2 Bacteria and infections

M. bovis BCG (strain Chicago) was cultured after mouse passage from spleens in Dubos broth base (Difco, Detroit, MI) supplemented with 10 % Dubos medium albumin (Difco). Bacteria were cultured to mid-log phase and, after brief sonification, they were aliquoted and stored at −70 °C until use. Bacteria were counted by plating serial tenfold dilutions on Middlebrook agar plates (Difco) supplemented with 10 % OADC enrichment (Difco). After 3 to 4 weeks of culture at 37 °C, bacterial colonies were counted. *M. bovis* BCG was thawed, washed with PBS, then inoculated at a dose of 2×10^6 to 6×10^6 viable bacteria in 0.2 ml PBS by i.v. injection via the lateral tail vein. At various time points after infection, lung, liver, and spleen were removed and homogenized with a labblender (Seward Medical, London, GB). Numbers of bacteria per organ were determined by plating tenfold serial dilutions of organ homogenates.

2.3 Stimuli *in vitro*

Mycobacterial proteins were prepared as described previously [19, 20] and used at the concentrations indicated in the figure legends. Briefly, for preparation of lysates, i.e. soluble somatic proteins, *M. tuberculosis* H37Rv bacteria were grown in Dubos broth base supplemented with 10 % Dubos medium albumin until mid-log phase [20]. Bacteria were sedimented, washed with PBS and disrupted with a glass bead mill (Braun, Melsungen, FRG). Insoluble membrane components were removed by ultracentrifugation (300 000 × g; 1 h) and supernatants were stored at −70 °C until use. For culture filtrates, i.e. mainly secreted proteins of mycobacteria, *M. tuberculosis* H37Rv organisms were grown in Sauton medium (Difco) for 18 days, sedimented, and the supernatants sterile-filtered [19]. Culture filtrates were concentrated 50-fold by ultrafiltration (Amicon, Danvers, MA) with a membrane of 5000 Da pore-size cut-off. In both preparations, a cocktail of protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 200 μM PMSF; Boehringer Mannheim, Mannheim, FRG) was included and the supernatant was sterile-filtered twice through 0.22 μm filters (Schleicher & Schüll, Dassel, FRG). Protein concentrations were determined with the bicinchonic acid (BCA) assay (Pierce, Rockford, IL). Heat-killed *M. bovis* BCG were prepared by autoclaving a mid log-phase culture of *M. bovis* BCG after washing twice with PBS. *M. tuberculosis* purified protein derivate (PPD) was obtained from Statens Serum Institute (Copenhagen, Denmark) and diluted in PBS. Freeze-dried *M. tuberculosis* H37Ra was obtained from Difco and diluted in Iscove's modified Dulbecco's medium (IMDM; Seromed, Berlin, FRG).

2.4 IFN-γ production

Mice were infected with *M. bovis* BCG as described above, and at the indicated time points, animals were killed and spleens removed. Single-cell suspensions were obtained by teasing the spleens through a stainless steel mesh and lysing erythrocytes with ammonium chloride. The cells were resuspended in IMDM supplemented with 10 % FCS (Boehringer Mannheim, Mannheim, FRG), 2 mM glutamine, 100 U/ml penicillin/streptomycin (Gibco, Paisley, GB) and 1 μg/ml indomethacin (Sigma, St. Louis, MO). The cells were seeded in 96-well plates (Nunc, Roskilde, Denmark) at a final concentration of 2×10^5 per well and cultured at 37 °C, 7 % CO_2 in a humidified incubator (Hereaus, FRG). Control cultures were stimulated with concanavalin A (Con A) as mitogen (Sigma). After 2 days of culture, the supernatants were removed and frozen at −20 °C until analyzed for IFN-γ concentrations by ELISA. All stimulation groups were analyzed in triplicate. The data shown represent one experiment repeated at least twice.

2.5 ELISA

Culture supernatants were screened for IFN-γ by double-sandwich ELISA using two specific monoclonal antibodies (mAb) recognizing different epitopes of the cytokine [21]. The second-step mAb was biotinylated to allow binding of streptavidin-conjugated alkaline phosphatase (Dianova, Hamburg, FRG) and detection with *p*-nitrophenyl phosphate (Sigma). Reactions were stopped with 0.5 M EDTA and optical densities were measured with an Intermed NJ-2000 Immunoreader (Nunc). ELISA LITE software (Meddata Inc., New York, NY) was used to calculate cytokine concentrations. Murine recombinant (r) IFN-γ was diluted in the medium described above to obtain a

standard curve. Detection limit of the ELISA was 0.05 U/ml IFN-γ. Rat anti-murine IFN-γ hybridomas R4-6A2 [22] and AN18-17.24 [23] were kindly provided after subcloning by Dr. J. Langhorne, Max-Planck-Institute for Immunobiology, Freiburg, FRG. Murine rIFN-γ with a specific activity of 10^7 U/mg was a generous gift of Dr. G. Adolf, Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria.

2.6 Flow cytometry

Spleen cells were stained with the following antibodies: anti-L3T4-PE (clone YTS 191) (Gibco), anti-Lyt2-FITC (clone YTS 169) (Gibco), anti-TcRα/β-biotin (clone H57-597, a kind gift from Dr. R. Kubo), anti-TcRγ/δ-biotin (clone GL-3, kindly provided by Dr. L. Lefrançois), anti-CD3-FITC (clone 145-2c11, a kind gift of J. Bluestone), anti-NK1.1-biotin (clone PK 136; ATCC, Rockville, MD), anti-Ly5(B220)-FITC, (clone RA3-6B2; Medac, Hamburg, FRG). Biotinylated antibodies were detected with streptavidin-phycoerythein conjugate (PE) (Gibco) or strepavidin-RED670 (Gibco). Cells were analyzed in a FACScan (Becton Dickinson, Mountain View, CA) using the LYSIS II software (Becton Dickinson).

2.7 DTH reactions

Mice were injected with 5 µg *M. tuberculosis* PPD in PBS in the left hind foodpad, and the swelling of the injected and contralateral foodpads were measured with a calipermeter after 48 h. The results are given as the difference between injected and contralateral foodpad. Non-infected mice were treated in the same way as controls for nonspec[illegible] swelling. Nonspecific reactions were not detected in th[illegible] experiments.

2.8 Histology

Livers from infected mice were snap-frozen in liqu[illegible] nitrogen. Sections of 10 µm thickness were cut in a Frigoc[illegible] cryotom (Reichert-Jung, Heidelberg, FRG) and stain[illegible] with hematoxylin-eosin stain (HE). Photomicrography w[illegible] performed with on Olympus photomicroscope.

3 Results

3.1 Phenotype analyses of spleen cells from *M. bovis* BCG-infected α/β and γ/δ T cell-deficient mutant mice

TcR-$\beta^{-/-}$, TcR-$\delta^{-/-}$, and RAG-$1^{-/-}$ mutants and thei[illegible] heterozygous controls were infected with *M. bovis* BCG and their spleen cells characterized phenotypically by flow cytometry at various time points (Table 1). Since data from the various heterozygous control mice differed minimally from each other, only results from TcR-$\beta^{+/-}$ mice are depicted in Table 1. In these controls, absolute numbers and relative proportions of α/β T cells gradually increased during *M. bovis* BCG infection with an almost stable 2:1 ratio of CD4 to CD8 α/β T cells. In heterozygous controls, numbers of γ/δ T cells steadily increased until day 30 p.i., and rapidly declined thereafter to normal values. NK1.1 cells increased transiently during the first month of infection. In the TcR-$\delta^{-/-}$ mutants, numbers of α/β T cells,

Table 1. Cytofluorometric analysis of spleen cells from *M. bovis* BCG-infected T cell mutant mice

Type of mice	Day of testing	% Gated cells (absolute numbers of positive spleen cells)[a]			
		CD3/TcR-αβ	CD3/TcR-γδ	NK1.1	Ly-5/B220
TcR-$\beta^{+/-}$	0	21.0 (3.6 × 10^7)	1.3 (0.2 × 10^7)	4.6 (0.8 × 10^7)	70.8 (12.1 × 10^7)
	15	21.9 (28.5 × 10^7)	2.9 (3.8 × 10^7)	5.8 (7.5 × 10^7)	39.8 (51.7 × 10^7)
	30	26.9 (21.5 × 10^7)	5.3 (4.2 × 10^7)	9.3 (7.4 × 10^7)	24.7 (19.8 × 10^7)
	60	36.3 (8.4 × 10^7)	5.1 (1.4 × 10^7)	5.1 (1.4 × 10^7)	55.9 (15.7 × 10^7)
	90	38.6 (8.5 × 10^7)	0.8 (0.2 × 10^7)	5.1 (1.1 × 10^7)	52.2 (11.5 × 10^7)
	120	46.8 (8.0 × 10^7)	1.2 (0.2 × 10^7)	6.4 (1.0 × 10^7)	40.1 (6.8 × 10^7)
TcR-$\beta^{-/-}$	0	n.d.[b]	9.2 (1.3 × 10^7)	3.3 (0.5 × 10^7)	83.8 (11.7 × 10^7)
	15	n.d.	9.7 (5.1 × 10^7)	8.4 (4.4 × 10^7)	61.2 (31.8 × 10^7)
	30	n.d.	15.7 (3.9 × 10^7)	6.9 (1.7 × 10^7)	69.5 (17.4 × 10^7)
	60	n.d.	12.9 (2.8 × 10^7)	7.0 (1.5 × 10^7)	84.1 (18.5 × 10^7)
	90	n.d.	6.9 (1.6 × 10^7)	10.3 (2.4 × 10^7)	57.7 (13.3 × 10^7)
TcR-$\delta^{-/-}$	0	31.5 (5.4 × 10^7)	n.d.	3.3 (0.6 × 10^7)	55.1 (9.4 × 10^7)
	15	27.5 (24.2 × 10^7)	n.d.	6.3 (5.5 × 10^7)	38.4 (3.8 × 10^7)
	30	41.7 (14.2 × 10^7)	n.d.	4.7 (1.6 × 10^7)	45.6 (15.5 × 10^7)
	60	52.0 (18.7 × 10^7)	n.d.	4.4 (1.6 × 10^7)	40.3 (14.5 × 10^7)
	90	38.8 (13.2 × 10^7)	n.d.	5.6 (1.8 × 10^7)	45.4 (15.4 × 10^7)
	120	42.4 (7.2 × 10^7)	n.d.	4.4 (0.8 × 10^7)	41.6 (7.1 × 10^7)
RAG-$1^{-/-}$	0	n.d.	n.d.	56.1 (2.0 × 10^7)	n.d.
	15	n.d.	n.d.	18.4 (0.6 × 10^7)	n.d.
	30	n.d.	n.d.	23.1 (0.8 × 10^7)	n.d.
	60	n.d.	n.d.	61.2 (0.3 × 10^7)	n.d.

a) Gated on "lymphoid cells" based on forward and side scatter, and calculated numbers from total isolated spleen cells.
b) n.d., not detectable

NK1.1 cells and B220 B cells were comparable to those in heterozygous control littermates throughout the course of infection. Thus, the loss of γ/δ T cells did not affect the quantity of the remaining lymphocyte populations. Although γ/δ T lymphocytes were increased in uninfected TcR-$\beta^{-/-}$ mutants, approximately equal numbers were observed in infected TcR-$\beta^{-/-}$ and TcR-$\beta^{+/-}$ mice. Throughout the infection, NK1.1 cells and B220 B cells were comparable in homozygous and heterozygous TcR-β mutants. In uninfected RAG-$1^{-/-}$ mice, relative numbers of NK1.1 cells were slightly increased compared to heterozygous controls, and infection marginally affected the quantity of NK1.1 cells in these mutants.

3.2 *M. bovis* BCG infection in α/β and γ/δ T cell-deficient mutant mice

A major goal of this study was the determination of the impact of deficiencies in defined T cell populations on resistance to *M. bovis* BCG. To this end, TcR-$\beta^{-/-}$, TcR-$\delta^{-/-}$ and RAG-$1^{-/-}$ mutants and their heterozygous controls were infected with *M. bovis* BCG, and CFU in spleens, livers and lungs determined at various time points thereafter (Fig. 1). Initial experiments revealed that RAG-$1^{-/-}$, but not TcR-$\beta^{-/-}$ nor TcR-$\delta^{-/-}$ mutants, succumbed to infection with more than 3×10^6 *M. bovis* BCG within 4 weeks. Therefore, the RAG-$1^{-/-}$ mutants, and their heterozygous littermates were infected with 2×10^6 *M. bovis* BCG organisms, whereas the TcR-β- and TcR-δ-heterozygous and homozygous mutants were infected with 6×10^6 mycobacteria. Fig. 1 shows the growth behavior of *M. bovis* BCG in the different mouse strains over the 120-day study period. Although the mycobacterial load in RAG-$1^{-/-}$ mutants was initially reduced, all mice died between day 60 and 90 p.i. Similarly, the TcR-$\beta^{-/-}$ mutant mice failed to control *M. bovis* BCG infection satisfactorily, although their survival time was prolonged until day 9 p.i. Whereas heterozygous controls succeeded in gradually reducing bacterial numbers to almost background levels during the 120 days studied, the bacterial load remained relatively constant in spleens and livers of TcR-$\beta^{-/-}$ mutants. Numbers of *M. bovis* BCG differed significantly in spleens and livers of homozygous *versus* heterozygous littermates as early as day 30 p.i. ($p < 0.05$, Student's *t*-test). The TcR-$\delta^{-/-}$ mutants were the only T cell-deficient mice to survive for the whole 120 day period. Although numbers of *M. bovis* BCG gradually declined in these mice, CFU were slightly elevated at later time points and, at day 120 p.i., showed a statistically significant increase in spleens and livers of homozygous *versus* heterozygous littermates ($p < 0.05$, Student's *t*-test). We conclude, first, that α/β T cells are essential for satisfactory control of *M. bovis* BCG, and that their loss results in fatal dissemination of *M. bovis* BCG. Second, although our data show efficient containment of *M. bovis* BCG organisms within infected organs in the absence of γ/δ T cells, they indicate a contribution of γ/δ T cells to the optimum control at late stages of BCG infection. Finally, it remains to be established whether the higher sensitivity to *M. bovis* BCG of RAG-$1^{-/-}$ mutants compared to TcR-$\beta^{-/-}$ mutants relates to the additional deficiency in B cells, γ/δ T cells, or both lymphocyte populations, in the former mice.

3.3 DTH reactions in *M. bovis* BCG-infected α/β and γ/δ T cell-deficient mutant mice

DTH reactions to PPD are common sequelae of *M. bovis* BCG vaccination and *M. tuberculosis* infection [1, 2]. We, therefore, followed DTH to PPD in the immunodeficient mutant mice during *M. bovis* BCG infection. As expected,

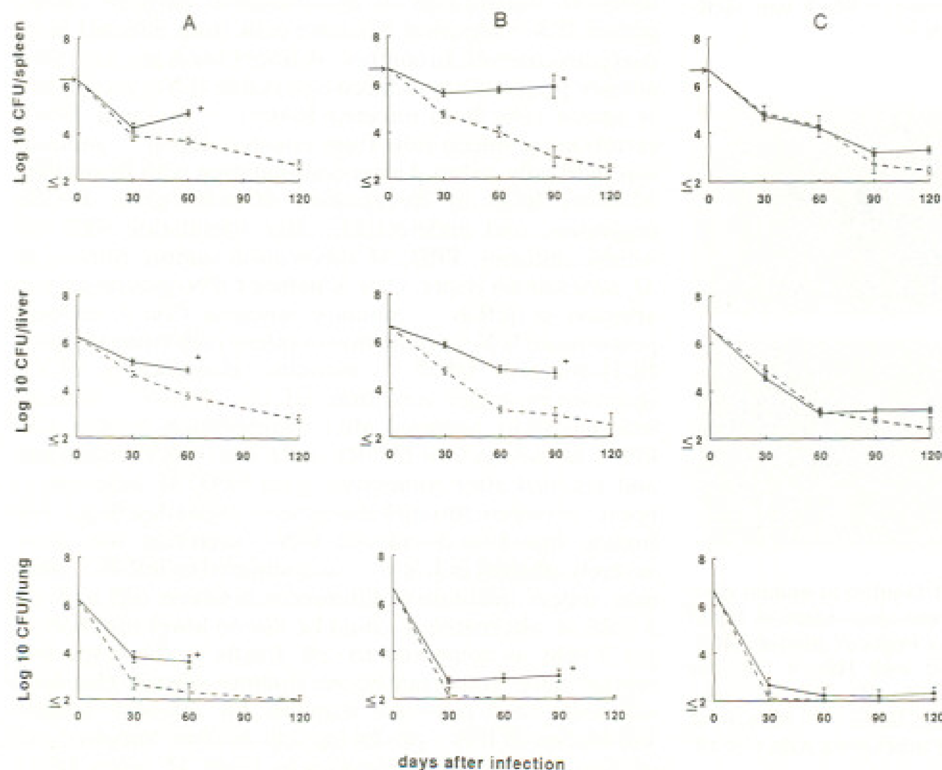

Figure 1. Growth of *M. bovis* BCG in organs of infected mutant mice. Mice were infected with *M. bovis* by i.v. injection. Organs were homogenized, serial tenfold dilutions were plated on Middlebrook agar plates and CFU counted 3–4 weeks later. Results are given as mean ± SD of four mice per time point of one experiment, repeated at least twice. RAG-$1^{-/-}$ and TcR-$\beta^{-/-}$ died after the indicated time points (+). (A) RAG-$1^{-/-}$ (●) and RAG$^{+/-}$ (○) mice, inoculum 2×10^6 *M. bovis* organisms/mouse, about one-third the dose of TcR-β and TcR-δ mice; (B) TcR-$\beta^{-/-}$ (●) and TcR-$\beta^{+/-}$ (○) mice, inoculum 6×10^6 *M. bovis* organisms/mouse; (C) TcR-$\delta^{-/-}$ (●) and TcR-$\delta^{+/-}$ (○) mice, inoculum 6×10^6 *M. bovis* organisms/mouse.

strong DTH reactions developed in *M. bovis* BCG-infected heterozygous control mice in response to PPD challenge (Fig. 2). In contrast, DTH reactions were totally absent in RAG-$1^{-/-}$ mutants and virtually undetectable in TcR-$\beta^{-/-}$ mutants. In TcR-$\delta^{-/-}$ mutants, DTH reactions were slightly reduced by day 30 and showed remarkable variation at later time points.

3.4 Granuloma formation in *M. bovis* BCG-infected α/β and γ/δ T cell-deficient mutant mice

The development of granulomatous lesions at the site of microbial growth is essential for local containment and successful eradication of pathogenic mycobacteria [1, 2]. In livers of heterozygous controls, organoid granulomas were observed by day 30 p.i. (data not shown) which had fu developed by day 60 p.i. (Fig. 3A). Frequently, the lesions contained a necrotic center without apparent sig of caseation. Granulomatous lesions also developed TcR-$\delta^{-/-}$ mutants, frequently with a slightly more diffu appearance (Fig. 3B). In contrast, livers of *M. bovis* BC infected TcR-$\beta^{-/-}$ and RAG-$1^{-/-}$ mutants were virtual devoid of lesions by day 30 p.i. (data not shown). By day p.i., small lymphoid accumlations were detected in son areas of infected livers of these mutants, mainly in th vicinity of blood vessels (Fig. 3C, D). Infrequently, infiltr tions of the liver parenchyma by inflammatory cells we observed in TcR-$\beta^{-/-}$ and RAG-$1^{-/-}$ mutants. Thus, ou data confirm the central role of α/β T cells in the formatio of mycobacterial granulomas and point to an auxiliar function of γ/δ T cells.

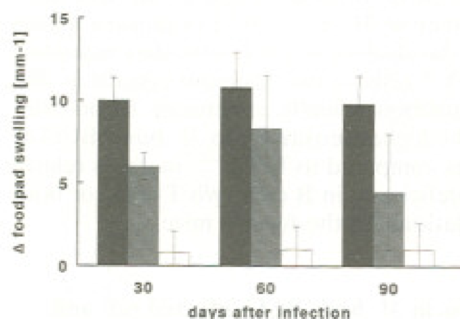

Figure 2. DTH reactions of *M. bovis* BCG-infected mutant mice. Infected mice were injected intradermally with 5 µg PPD in PBS into the left hind footpad and the dorsoventral thickness of both hind footpads was determined 48 h later with a calipermeter. Results are given as the difference between the injected *vs.* noninjected foodpad. Mean ± SD of four mice per time point. Uninfected control mice (TcR-$\beta^{+/-}$) showed $\Delta < 1\ \mathrm{mm}^{-1}$. In RAG-$1^{-/-}$ no foodpad swelling was observed. Black bars: TcR-$\beta^{+/-}$; open bars: TcR-$\beta^{-/-}$; gray bars TcR-$\delta^{-/-}$.

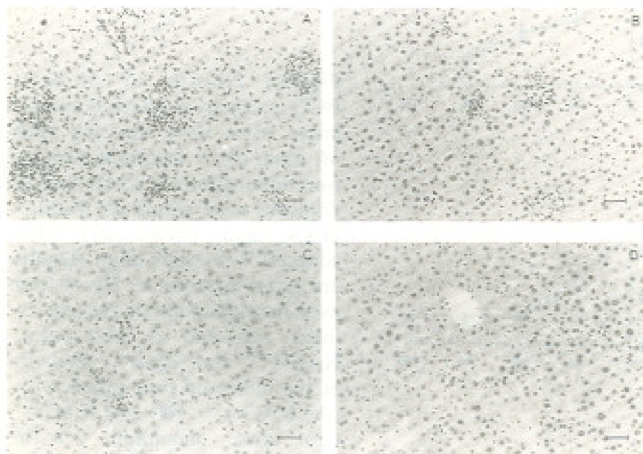

Figure 3. Liver histology of *M. bovis* BCG-infected mutant mice at day 60. Livers from infected mice were snap-frozen in liquid nitrogen. Serial sections were cut with a Frigocut cryotom (Reichert-Jung, FRG). Slides were stained with HE. × 170; bar represents 40 µm. (A) TcR-$\beta^{+/-}$ control; (B) TcR-$\delta^{-/-}$; (C) TcR-$\beta^{-/-}$; (D) RAG-$1^{-/-}$. Note that the RAG-$1^{-/-}$ mice were infected with only 2×10^6 *M. bovis*; all other mice with 6×10^6 *M. bovis*.

3.5 Cytokine secretion after stimulation *in vitro* with different mycobacterial antigen preparations by spleen cells from *M. bovis* BCG-infected α/β and γ/δ T cell-deficient mutant mice

IFN-γ is the central cytokine in acquired resistance to tuberculosis [3]. Therefore, the capacity of spleen cells from *M. bovis* BCG-infected α/β and γ/δ T cell-deficient mice to produce IFN-γ after stimulation with various mycobacterial antigen preparations was assessed over the course of infection (Fig. 4). The T cell mitogen Con A (1 µg/ml) was included in these experiments. The antigen preparations used included heat-killed *M. bovis* BCG organisms (10^6) and freeze-dried *M. tuberculosis* (5 µg/ml) as a source of particulate somatic antigens; *M. tuberculosis* H37Rv lysates (5 µg/ml) as a source of solubilized somatic antigens; PPD and culture filtrates of *M. tuberculosis* (both used at a concentration of 5 µg/ml) as a source of secreted antigens. Not only the mitogen Con A, but also the different preparations of mycobacterial antigens caused potent IFN-γ secretion in spleen cells from infected heterozygous controls. In contrast, neither Con A nor any of the antigen preparations induced detectable IFN-γ production in spleen cells from infected RAG-$1^{-/-}$ mutants. IFN-γ secretion by spleen cells from infected TcR-$\beta^{-/-}$ mutants was markedly reduced after restimulation with heat-killed *M. bovis* BCG or freeze-dried *M. tuberculosis* H37Ra organisms, and undetectable after stimulation with the soluble antigens, PPD, *M. tuberculosis* culture filtrate, or *M. tuberculosis* lysate. Con A-induced IFN-γ secretion was affected in TcR-$\beta^{-/-}$ mutants, whereas Con A induced pronounced IFN-γ production in spleen cells from *M. bovis* BCG-infected TcR-$\delta^{-/-}$ mutants. Remarkably, IFN-γ secretion by spleen cells from infected TcR-$\delta^{-/-}$ mutants was drastically impaired after restimulation *in vitro* with killed *M. tuberculosis* H37Ra or *M. bovis* BCG organisms and minimal after stimulation with PPD, *M. tuberculosis* lysate, or culture filtrate between day 30 and day 90 p.i. The finding that Con A-induced IFN-γ secretion was more severely affected in TcR-$\beta^{-/-}$ as compared to TcR-$\delta^{-/-}$ mice may reflect qualitative differences between α/β and γ/δ T cells or, alternatively, could be due to lower numbers of γ/δ T cells as compared to α/β T cells in the respective mutant mice. Importantly, our findings suggest that both α/β and γ/δ T cells are required for efficient antigen stimulation of IFN-γ producing cells *in vitro*. Supernatants of Con A-stimulated spleen cells from *M. bovis* BCG-

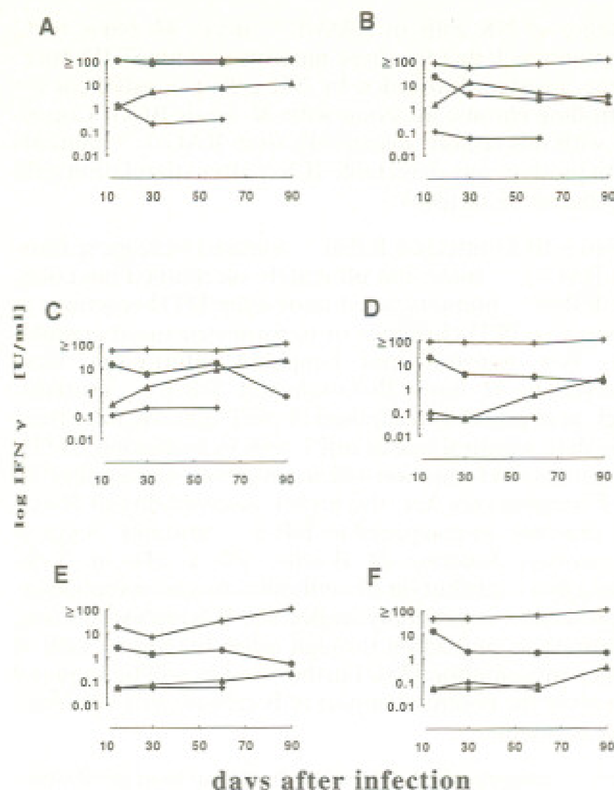

Figure 4. IFN-γ secretion by spleen cells from *M. bovis* BCG-infected mutant mice. Spleen cells (2×10^5 per well) from infected animals were cultured for 2 days with the following antigens or mitogen. (A) Con A (1 µg/ml); (B) *M. tuberculosis* H37Ra (5 µg/ml); (C) heat-killed *M. bovis* BCG (10^6); (D) PPD (5 µg/ml); (E) *M. tuberculosis* lysate (somatic antigens; 5 µg/ml); (F) *M. tuberculosis* culture filtrate (secreted antigens; 5 µg/ml). Note logarithmic scale. IFN-γ concentrations were measured by ELISA as described in Sect. 2.5; the detection limit was 0.05 U/ml. Results represent the mean of triplicates of a representative experiment (repeated at least once); SD < 10 %; (+) control; (▲) TcR-$\beta^{-/-}$; (●) TcR-$\delta^{-/-}$; (◆) RAG-$1^{-/-}$.

infected TcR-$\beta^{-/-}$, TcR-$\delta^{-/-}$ or control mice contained only minimal IL-4 (data not shown). These findings suggest that lowered IFN-γ secretion in TcR-$\delta^{-/-}$ mutants did not favor the development of T_H2 cells.

3.6 Reconstitution of IFN-γ production *in vitro*

The data presented in Fig. 4 underline the need for both α/β and γ/δ T cells in optimum IFN-γ secretion *in vitro*. In an attempt to reconstitute deficient IFN-γ production, equal numbers of spleen cells from *M. bovis* BCG-infected TcR-$\beta^{-/-}$ and TcR-$\delta^{-/-}$ mutants were mixed and restimulated with mycobacterial antigen preparations. As shown in Fig. 5, the IFN-γ response was partially (day 30 p.i.) or fully (day 90 p.i.) reconstituted in these cell mixtures. IFN-γ secretion was even re-established when spleen cells from non-infected TcR-$\delta^{-/-}$ or TcR-$\beta^{-/-}$ mice were added to spleen cells from *M. bovis* BCG-infected TcR-$\beta^{-/-}$ or TcR-$\delta^{-/-}$ mutants, respectively. In contrast, combination of spleen cells from uninfected TcR-$\beta^{-/-}$ and TcR-$\delta^{-/-}$ mice did not result in successful reconstitution. We conclude from these data that competence to synthesize IFN-γ was induced in the respective T lymphocyte populations remaining in TcR-$\beta^{-/-}$ or TcR-$\delta^{-/-}$ mutants during *M. bovis* BCG infection. However, successful IFN-γ secretion *in vitro* not only depended upon antigen-specific stimulation of both T cell subsets, but also required regulatory interactions with the alternate T cell population which were probably independent of the nominal mycobacterial antigen.

4 Discussion

The intracellular residence of mycobacteria in macrophages is crucial for their persistence in the host [1, 2, 24, 25]. Specific T lymphocytes promote protection through at least two major mechanisms. First, they induce the formation of granulomatous lesions which are essential for microbial containment to distinct foci and, second, they

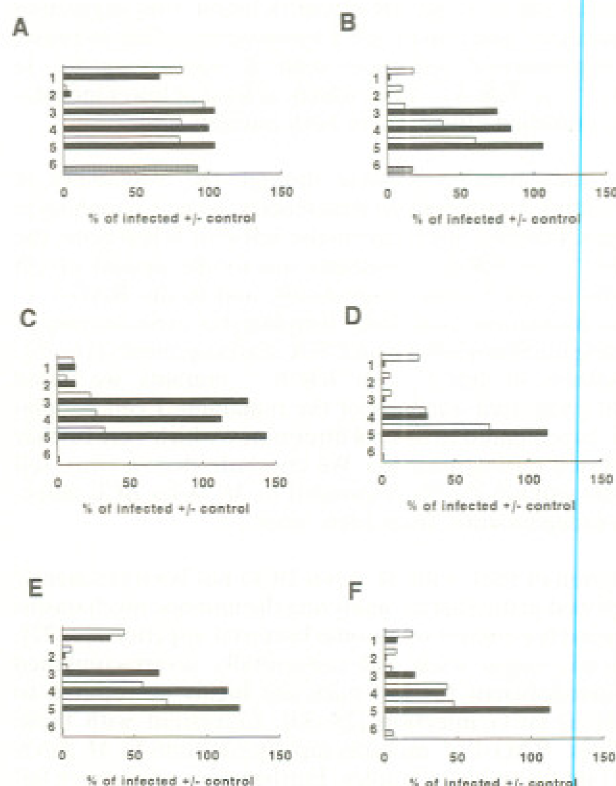

Figure 5. Reconstituted IFN-γ secretion by mixed spleen cel[ls] from *M. bovis* BCG-infected TcR-$\delta^{-/-}$ and TcR-$\beta^{-/-}$ mutant mic[e]. Equal numbers of spleen cells from *M. bovis* BCG-infected o[r] uninfected animals were mixed and co-cultured for 2 days with th[e] antigens indicated in the legend to Fig. 4. The figure shows percen[t] IFN-γ produced by 2×10^5 spleen cells from infected heterozygou[s] controls. (1) Infected TcR-$\delta^{-/-}$ (2×10^5 cells); (2) infecte[d] TcR-$\beta^{-/-}$ (2×10^5 cells); (3) noninfected TcR-$\delta^{-/-}$ (1×10^5 cell[s]) + infected TcR-$\beta^{-/-}$ (1×10^5 cells); (4) infected TcR-$\delta^{-/-}$ (1×10^5 cells) + infected TcR-$\beta^{-/-}$ (1×10^5 cells); (5) infected TcR-$\delta^{-/-}$ (1×10^5 cells) + noninfected TcR-$\beta^{-/-}$ (1×10^5 cells); (6) noninfected TcR-$\delta^{-/-}$ (1×10^5 cells) + noninfected TcR-$\beta^{-/-}$ (1×10^5 cells). (A) Con A; (B) *M. tuberculosis* H37Ra; (C) Heat-kille[d] *M. bovis* BCG; (D) PPD; (E) *M. tuberculosis* lysate; (F) *M. tuberculosis* culture filtrate. Open bars: data from day 30 p.i.; blac[k] bars: data from day 90 p.i.; hatched bar from mixed, non-infecte[d] TcR-$\delta^{-/-}$ and TcR-$\beta^{-/-}$ mice.

macrophages. IFN-γ is central to both mechanisms, although other cytokines may be involved [3]. Consistent with these findings, IFN-γ-deficient mutant mice suffer severely from progressive infection with *M. tuberculosis* [5, 6]. Even after maximal T cell activation, pathogenic mycobacteria persist in the immunocompetent host, though in reduced numbers. As a consequence, chronic infection develops which is effectively controlled, but not eradicated by T lymphocytes. This contrasts to acute infection with the intracellular pathogen *L. monocytogenes*, which is fully eradicated as a consequence of T cell activation.

The aim of this study was to assess the relative contribution of α/β and γ/δ T cells to the host response against *M. bovis* BCG infection. Our data formally prove the central role of α/β T cells in the major aspects of T cell mediated immunity, namely mycobacterial growth inhibition, granuloma formation and DTH development *in vivo* as well as antigen-induced IFN-γ production *in vitro*. Moreover, our data reveal an effect of the TcR-δ gene deletion, thus suggesting an auxiliary function of γ/δ T lymphocytes. This contrasts to experimental infection with *L. monocytogenes* in TcR-$\beta^{-/-}$ or TcR-$\delta^{-/-}$ mice which, at least at lower inocula, was controlled efficiently in both mutants [26].

Our study became possible through the availability of mutant mice with defined deficiencies in the T lymphocyte system. Due to a disruption in the TcR-δ or TcR-β gene, the TcR-$\delta^{-/-}$ or TcR-$\beta^{-/-}$ mutants are totally devoid of γ/δ T cells or α/β T cells, respectively, and in the RAG-$1^{-/-}$ mice, no mature, functional lymphocytes exist because of missing immunoglobulin and TcR rearrangements [16–18]. In spleens of TcR-$\beta^{-/-}$ or TcR-$\delta^{-/-}$ mutants, we found slightly elevated numbers of the remaining T cell population, as compared to control littermates, which were further increased during infection. We conclude that *in vitro*, α/β T cells and γ/δ T cells responded to *M. bovis* BCG infection independently from each other.

Infection of mice with *M. bovis* BCG has been frequently employed in the past for analyzing the immune mechanisms required for control of chronic bacterial infections [9, 27]. Athymic nu/nu mice and congenitally severe-combined immunodeficient (SCID) mice are highly susceptible to *M. bovis* BCG infection [28–30]. Consistent with these findings, RAG-$1^{-/-}$ mutants rapidly succumb to *M. bovis* BCG infection in our studies. Furthermore, these mice fail to develop DTH reactions in response to PPD and to form lesions at the site of mycobacterial implantation. Together, these findings confirm the central role of lymphocytes in the acquisition of resistance to mycobacteria. The formation of granulomatous lesions in *M. bovis* BCG-infected SCID mice has been described [30], and our own experiments with SCID mice confirm this observation (data not shown). It is known that SCID mice have low levels of residual T and B cells which might be activated during mycobacterial infection [31, 32]. In contrast, RAG-$1^{-/-}$ mice are completely devoid of any functional T and B lymphocytes. Small numbers of T lymphocytes may be responsible for development of lesions in SCID mice, and do not argue against the strict lymphocyte dependence of granuloma formation. SCID mice are also relatively resistant to acute infection with *L. monocytogenes*, thought to be due to the presence of highly activated NK cells [33, 34]. Despite the presence of NK cells in RAG-$1^{-/-}$ mice, *M. bovis* BCG infection was lethal for these mutants and for SCID mice. Hence, resistance afforded by NK cells is insufficient fo controlling chronic infection with *M. bovis* BCG. Consistent with this notion, spleen cells from RAG-$1^{-/-}$ mutants fail to produce any detectable IFN-γ after stimulation with mycobacterial antigens.

M. bovis BCG-infected TcR-$\beta^{-/-}$ mutants were more resistant RAG-$1^{-/-}$ mice, but ultimately succumbed infection. The TcR-$\beta^{-/-}$ mutants failed to develop DTH reactions in response to PPD challenge or to form structured granulomas. Few nonstructured lymphoid infiltrations were observed in *M. bovis* BCG-infected TcR-$\beta^{-/-}$ mutants, which were probably composed of γ/δ T cells. We infer from these data a central role of α/β T cells in protection, DTH, and granuloma formation which cannot be compensated by γ/δ T lymphocytes. Yet, the higher susceptibility of RAG-$1^{-/-}$ mutants, as compared to TcR-$\beta^{-/-}$ mutants, suggests an auxiliary function of B cells, γ/δ T cells or both. Although a contribution of antibodies to anti-mycobacterial protection is generally neglected, B lymphocytes may contribute to protection through other functions, such as antigen presentation [35]. Further studies will be required to analyze the potential impact of B cells on anti-mycobacterial immunity.

Rag-$1^{-/-}$ mutants died with a low bacterial load in all three organs examined. Similarly, death, of TcR-$\beta^{-/-}$ mice was not accompanied by marked increase in bacterial numbers. Comparable findings have been recently described for MHC class I- or MHC class II-deficient mice infected with *M. bovis* BCG [36]. Neither RAG-$1^{-/-}$ nor TcR-$\beta^{-/-}$ mutants developed granulomatous lesions in response to *M. bovis* BCG infection. Perhaps the failure to contain bacteria within distinct foci facilitated uncontrolled dissemination of bacilli throughout infected organs, thus resulting in death caused by fewer microorganisms.

Although γ/δ T cells failed to compensate efficiently for the α/β T cell deficiency in protection against *M. bovis* BCG, our study suggests an auxiliary function of γ/δ T cells in anti-mycobacterial immunity. Indirect evidence for participation of γ/δ T cells in the immune response to mycobacteria has been presented previously. In these studies, mycobacterial components were shown to be potent stimulators of the major γ/δ T cell population in the peripheral blood of normal individuals, expressing the Vγ9δ2 chain combination [37]. This antigenic molecule, however, is a nonproteinacious, low molecular weight compound which contains phosphate as essential stimulatory moiety [37–40]. γ/δ T cells have been found to accumulate in some types of lesions during mycobacterial infections [13]. Furthermore, γ/δ T lymphocytes from normal mice are stimulated by mycobacterial heat-shock protein [41]. Immunization with killed mycobacteria and infection with viable *M. bovis* BCG or *L. monocytogenes* leads to their activation *in vivo* prior to α/β T cells [11, 12, 42, 43]. In the listeriosis model, evidence has been presented that γ/δ T cells promote early resistance to listeriosis, and in TcR-$\beta^{-/-}$ mutants, the γ/δ T cells have been shown to compensate partially for the loss of protective α/β T cells [26, 43].

In our experiments, γ/δ T cell-deficient mice were as resistant to *M. bovis* BCG as their control littermates

during the first 90 days of infection. Thereafter, however, a small though significant, increase in mycobacterial number was observed in spleens and livers. Evidence for a critical role of γ/δ T cells early in the antibacterial response has been presented [12, 26, 43]. The data presented here suggest participation of γ/δ T cells in late stages of protective immunity against *M. bovis* BCG. Consistent with a role for γ/δ T cells later in anti-mycobacterial immunity, lesions in TcR-$\delta^{-/-}$ mutants appeared to be normal early in infection and became more diffuse at later time points. In contrast, abscess-like lesions developed in TcR-$\delta^{-/-}$ mutants early after infection with *L. monocytogenes* [26].

Evidence is overwhelming that IFN-γ is central to protective immunity to mycobacterial infections [1, 3, 5, 6]. Selected α/β and γ/δ T cells from healthy individuals, as well as from immunocompetent mice, produce IFN-γ as well as other cytokines *in vitro* after stimulation with mycobacteria [3, 44–47]. Furthermore, the particulate mycobacterial antigen used here has been shown to stimulate γ/δ T cells from immunocompetent mice after immunization with mycobacteria [11]. In this study, high IFN-γ levels were stimulated by the T cell mitogen Con A in spleen cells from *M. bovis* BCG-infected TcR-$\delta^{-/-}$ mutants and, to a lower extent, in cells from infected TcR-$\beta^{-/-}$ mice. In contrast, antigen-induced IFN-γ production was impaired in spleen cell cultures from *M. bovis* BCG-infected mutants lacking either α/β or γ/δ T cells. IFN-γ secretion *in vitro* could be completely re-established by the mixture of spleen cells from TcR-$\beta^{-/-}$ and TcR-$\delta^{-/-}$ mutants. Reconstitution was even achieved when one of the two admixed cell populations was derived from uninfected mutants. In contrast, combination of spleen cells from non-infected TcR-$\beta^{-/-}$ and TcR-$\delta^{-/-}$ mice failed to re-establish IFN-γ secretion. Thus, our findings strongly suggest that, although either T cell population is able to produce IFN-γ after specific restimulation with mycobacterial antigen preparations, optimum IFN-γ secretion *in vitro* requires the presence of both α/β and γ/δ T cells. We assume that the competence to produce IFN-γ after specific restimulation with mycobacterial antigens is induced in α/β and γ/δ T cells, each independent of the alternate T cell population, during *M. bovis* BCG infection. However, in the absence of the respective T cell subset, neither α/β nor γ/δ T cells alone were efficiently triggered for IFN-γ secretion *in vitro* by mycobacterial antigens. Rather, it appears that full IFN-γ production depends on regulatory interactions between α/β and γ/δ T cells, which are independent of the nominal mycobacterial antigens. Evidence is emerging to suggest regulatory interactions between γ/δ T cells and α/β T cells [48–50]. Despite reduced IFN-γ secretion *in vitro*, small quantities were still measured: our findings, therefore, do not exclude a role for IFN-γ in protective immunity to mycobacterial infection.

Recent experiments have shown that resistance and susceptibility to various infections are strongly influenced by the relative contributions of IFN-γ-producing T_H1 cells and IL-4-secreting T_H2 cells to the immune response [51]. Further, the development of T_H1 and T_H2 cells is regulated by the respective cytokines IFN-γ and IL-4 [51–53]. In experimental mycobacterial infections, T_H1 cells generally dominate, while T_H2 cells are only marginally or not at all activated [2]. In our experiments, antigen-specific IL-4-producing T_H2 cells could not be detected in *M. bovis* BCG-infected TcR-$\delta^{-/-}$ mutants despite diminished IFN-γ production. Thus, at least in this infection model, reduced IFN-γ secretion did not directly promote development of IL-4-secreting T_H2 cells.

Tuberculosis remains a major health threat in developing nations and has dramatically increased in industrialized countries during recent years [54]. Chemotherapy has been dramatically complicated by the emergence of multi-drug resistant strains [54]. Therefore, the possibility of preventing tuberculosis by vaccination has regained considerable interest. Although the value of *M. bovis* BCG vaccination has been questioned, a recent meta-analysis statistical study suggests an efficacy of approximately 50% [55], suggesting that prevention by an improved vaccine, either on the basis of *M. bovis* BCG, or on an alternate carrier system, may become a feasible goal in the future. Our findings demonstrate that vaccine-induced protection not only crucially depends on α/β T cells, but also involves γ/δ T cells. Studies with immunodeficient mutant mice will not only provide deeper insights into the cellular mechanisms of anti-mycobacterial immunity, but may also help to elucidate the efficacy and risk of an improved vaccine against tuberculosis which is capable of inducing the appropriate combination of the T cell subsets required for long-lasting protection.

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