

# Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection

(tuberculosis/CD8 T cells/cytotoxic T lymphocytes/microbial immunity/ $\beta_2$ -microglobulin-negative mice)

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**ABSTRACT** Mice with a targeted disruption in the  $\beta_2$ -microglobulin ( $\beta_2m$ ) gene, which lack major histocompatibility complex class I molecules and consequently fail to develop functional CD8 T cells, provided a useful model for assessing the role of class I-restricted T cells in resistance to infection with virulent *Mycobacterium tuberculosis*. Of mutant  $\beta_2m^{-/-}$  mice infected with virulent  $10^6$  *M. tuberculosis*, 70% were dead or moribund after 6 weeks, while all control mice expressing the  $\beta_2m$  gene remained alive for >20 weeks. Granuloma formation occurred in mutant and control mice, but far greater numbers of tubercle bacilli were present in the lungs of mutant mice than in controls, and caseating necrosis was seen only in  $\beta_2m^{-/-}$  lungs. In contrast, no differences were seen in the course of infection of mutant and control mice with an avirulent vaccine strain, bacille Calmette–Guérin (BCG). Immunization with BCG vaccine prolonged survival of  $\beta_2m^{-/-}$  mice after challenge with *M. tuberculosis* for 4 weeks but did not protect them from death. These data indicate that functional CD8 T cells, and possibly T cells bearing  $\gamma\delta$  antigen receptor, are a necessary component of a protective immune response to *M. tuberculosis* in mice.

The reemergence of tuberculosis has added a new urgency to understanding the protective immune responses to this disease (1). Virtually all experimental data indicate that cell-mediated rather than humoral immunity is the essential immune response for resistance to *Mycobacterium tuberculosis* infection (2, 3), and that CD4 T cells are necessary to that resistance (4–6); it remains unclear whether they are sufficient. The roles of CD8 and  $\gamma\delta$  T cells in the control of tuberculosis are less well understood. Data on the question are conflicting, suggesting that either CD4 (4, 6, 7) or CD8 (7, 8) T cells are sufficient for protection, or that both subsets are necessary (5, 9).

The construction of a mouse which has a targeted disruption of the gene for  $\beta_2$ -microglobulin ( $\beta_2m^{-/-}$ ) (10) has allowed the development of a murine system in which to examine critically the role of CD8 T cells in *M. tuberculosis* infection. This deletion results in the failure to express functional class I major histocompatibility complex (MHC) molecules and, consequently, in a paucity of CD8 cells due to the absence of positive antigen selection. Because the  $\beta_2m^{-/-}$  mice are devoid of MHC class I-restricted T cells, including cytotoxic T lymphocytes (CTLs), we thought that they might represent a useful *in vivo* model in which to address the question of the role MHC class I-restricted T cells in *M. tuberculosis* infection.

## MATERIALS AND METHODS

**Mice.** Six- to eight-week-old C57BL/6 (National Cancer Institute), 129 (The Jackson Laboratory), and  $\beta_2m^{-/-}$  mice

(bred as homozygotes) were used. All mice were maintained in a specific-pathogen-free environment and were found to be free of all 12 mouse pathogens tested.

**Bacterial Strains and Mouse Infections.** Bacille Calmette–Guérin (BCG, Pasteur strain) and virulent *M. tuberculosis* (Erdman strain) were obtained from the Trudeau Institute (Lake Saranac, NY) and stored at  $-70^\circ\text{C}$  until use. BCG and *M. tuberculosis* cells in phosphate-buffered saline (PBS) containing 0.05% Tween 80 were briefly sonicated before injection into mice ( $10^6$  bacteria per mouse, i.v.).

**Organ Harvesting.** Lungs, spleens, and livers were harvested from mice, homogenized in PBS/0.05% Tween 80 with a Stomacher homogenizer (Tekmar, Cincinnati), and plated on supplemented 7H10 medium (Difco). For frozen sections, tissues were embedded in OCT (Miles), flash frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$ .

**Histology.** Sections ( $5\ \mu\text{m}$ ) from paraffin blocks containing lung, liver, and spleen were cut and stained with hematoxylin and eosin or by the Ziehl–Neelsen method for acid-fast bacilli (AFB). The mean number of AFB was evaluated by counting the organisms within granulomas in 20 random  $\times 400$  fields for each section. Liver and spleen sections were evaluated for granulomas per 10 random  $\times 100$  fields. Ten random granulomas were examined for mean number of AFB.

**Immunohistochemistry.** Frozen  $5\text{-}\mu\text{m}$  sections of lung were mounted on poly(L-lysine)-coated slides and fixed in cold acetone. Sections were blocked with rabbit serum and incubated at  $4^\circ\text{C}$  with rat anti-mouse CD4 or CD8 monoclonal antibodies ( $10\ \mu\text{g}/\text{ml}$ ). The secondary antibody was biotinylated rabbit anti-rat IgG, and the staining was by the avidin-biotin-peroxidase complex method with diaminobenzidine as substrate.

**Cytokine Assays.** Spleen cells from mice 2–3 weeks post-infection were stimulated with either Con A ( $5\ \mu\text{g}/\text{ml}$ ), tuberculin purified protein derivative (PPD,  $10\ \mu\text{g}/\text{ml}$ ), or *M. tuberculosis* sonicate ( $10\ \mu\text{g}/\text{ml}$ ). Culture supernatants harvested at 48 hr and sera (from five mice) were filtered through  $0.45\text{-}\mu\text{m}$  filters, and interferon  $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) production determined by ELISA (Genzyme).

## RESULTS

***M. tuberculosis* Infection in  $\beta_2m^{-/-}$  and Control Mice.** Mice with a disruption in both copies of the  $\beta_2m$  gene (10) were backcrossed to the C57BL/6 background.  $\beta_2m^{-/-}$  mice and C57BL/6 control mice were infected with  $10^6$  *M.*

Abbreviations: AFB, acid-fast bacilli; CTL, cytotoxic T lymphocyte; BCG, bacille Calmette–Guérin;  $\beta_2m$ ,  $\beta_2$ -microglobulin; MHC, major histocompatibility complex; cfu, colony-forming units; PPD, tuberculin purified protein derivative; IFN, interferon; TNF, tumor necrosis factor.

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*tuberculosis* bacteria i.v. By 6 weeks, 70% of the  $\beta_2m^{-/-}$  mice were dead or moribund (Fig. 1). In contrast, all C57BL/6 mice survived >20 weeks. In histological sections, the number of AFB per field was consistently 10–1000 times higher in  $\beta_2m^{-/-}$  mice than in C57BL/6 mice. Colony counts from spleen, liver, and lungs of the mice did not consistently reflect the number of AFB seen in histological sections, although at later time points a 10-fold difference in colony-forming units (cfu) was seen between  $\beta_2m^{-/-}$  and C57BL/6 mice (Fig. 2a). We attribute this inconsistency to technical difficulties in disrupting infected cells in organs without creating an aerosol.

Histological sections of lung tissue from both  $\beta_2m^{-/-}$  and control mice had well-demarcated granulomas composed of clusters of epithelioid macrophages with intervening collections of lymphocytes at 3 weeks postinfection. The morphology of the granulomas in the control and  $\beta_2m^{-/-}$  mice was very similar, but lung sections from  $\beta_2m^{-/-}$  mice showed significantly more AFB inside the granulomas than those from C57BL/6 mice (Fig. 3). Macrophages were frequently completely full of AFB in the  $\beta_2m^{-/-}$  lung sections, whereas the C57BL/6 macrophages generally contained 1–5 AFB per infected macrophage. By 6 weeks postinfection 70% of the lung volume was taken up by merging granulomas in the sections from  $\beta_2m^{-/-}$  mice, compared with  $\approx 30\%$  in the control mouse sections.  $\beta_2m^{-/-}$  sections contained CD4-positive cells within the granulomas and alveolar spaces, but CD8-staining cells were absent. Granulomas from the control mice stained positively for both CD4 and CD8 cells (Fig. 3). Histological examination of the liver and spleen from  $\beta_2m^{-/-}$  and control mice showed well-formed granulomas, with a central core of epithelioid macrophages rimmed by lymphocytes.  $\beta_2m^{-/-}$  liver and spleen sections showed an  $\approx 10$ -fold increase in AFB compared with the control.

When  $\beta_2m^{-/-}$  mice judged to be within 4–5 days of death were sacrificed for histology, the lung sections revealed large confluent necrotizing granulomas occupying 60–80% of the lung volume. Necrosis and extracellular AFB were prominent within the center of the granulomas, whereas the peripheral zone consisted of apparently viable macrophages containing numerous intracellular AFB, lymphocytes, and neutrophils. Most strikingly, necrosis was not seen in control mice.

Although the  $\beta_2m^{-/-}$  mice had been backcrossed to a C57BL/6 background, the progeny still contained some genes of the 129 parental strain. C57BL/6 mice, the backcross background strain, were routinely used as control ( $\beta_2m^{+/+}$ ) mice. To test the possibility that the strain 129

genes present in the  $\beta_2m^{-/-}$  mice might affect the outcome of a *M. tuberculosis* infection, we compared infection of both parental strains, C57BL/6 and 129, with infection of the  $\beta_2m^{-/-}$  mice. Strain 129 mice were similar to C57BL/6 mice with respect to cfu and mortality following infection (data not shown). Thus, it appeared that the uncontrolled *M. tuberculosis* growth and mortality seen in the infected  $\beta_2m^{-/-}$  mice was primarily the result of the defect in class I expression, and not of the 129 background.

**BCG Infection in  $\beta_2m^{-/-}$  and Control Mice.** BCG, the avirulent *Mycobacterium bovis* vaccine strain, is frequently used as an experimental model for *M. tuberculosis* infection. Following infection of  $\beta_2m^{-/-}$  and C57BL/6 mice with BCG, no mortality or differences in cfu from the spleen, liver, or lungs among the strains of mice were observed up to 28 weeks postinfection (Fig. 2c). In lung sections taken from  $\beta_2m^{-/-}$  and C57BL/6 mice immunized with BCG for 8 weeks, well-formed granulomas and very few AFB were seen. In contrast to infection with virulent *M. tuberculosis*, the course of BCG infection was not altered perceptibly by the absence of CD8 T cells.

***M. tuberculosis* Infection Following BCG Immunization in  $\beta_2m^{-/-}$  Mice.** We explored whether immunization with BCG could engender protection in  $\beta_2m^{-/-}$  mice. Both  $\beta_2m^{-/-}$  and C57BL/6 mice were immunized with BCG vaccine 8 weeks prior to challenge with *M. tuberculosis*. By 9 weeks after the *M. tuberculosis* challenge, 70% of the  $\beta_2m^{-/-}$  mice had died (Fig. 1). In contrast, all of the C57BL/6 mice survived >20 weeks. At 10 weeks postinfection,  $\beta_2m^{-/-}$  lungs showed a 10-fold increase in *M. tuberculosis* cfu relative to C57BL/6 lungs (Fig. 2b). Granulomas from both  $\beta_2m^{-/-}$  and C57BL/6 mice appeared morphologically similar, and AFB from lung sections showed a 100-fold increase in bacterial load in the  $\beta_2m^{-/-}$  mice compared with control mice. Thus, immunization with BCG increased survival time for *M. tuberculosis*-infected  $\beta_2m^{-/-}$  mice by 3–5 weeks but did not protect the mice from death.

**Cytokine Production in  $\beta_2m^{-/-}$  Mice Infected with *M. tuberculosis*.** Both IFN- $\gamma$  and TNF- $\alpha$  are required for activation of macrophages to produce reactive nitrogen intermediates and to kill ingested *M. tuberculosis* (11–13). Since CD8 T cells are known to make IFN- $\gamma$ , the production of those cytokines was examined in  $\beta_2m^{-/-}$  and control mice infected with *M. tuberculosis*. IFN- $\gamma$  and TNF- $\alpha$  production did not differ significantly between spleen cells from  $\beta_2m^{-/-}$  and C57BL/6 mice stimulated *in vitro* with Con A or *M. tuberculosis* sonicate, although stimulation with PPD resulted in a slight increase in both IFN- $\gamma$  and TNF- $\alpha$

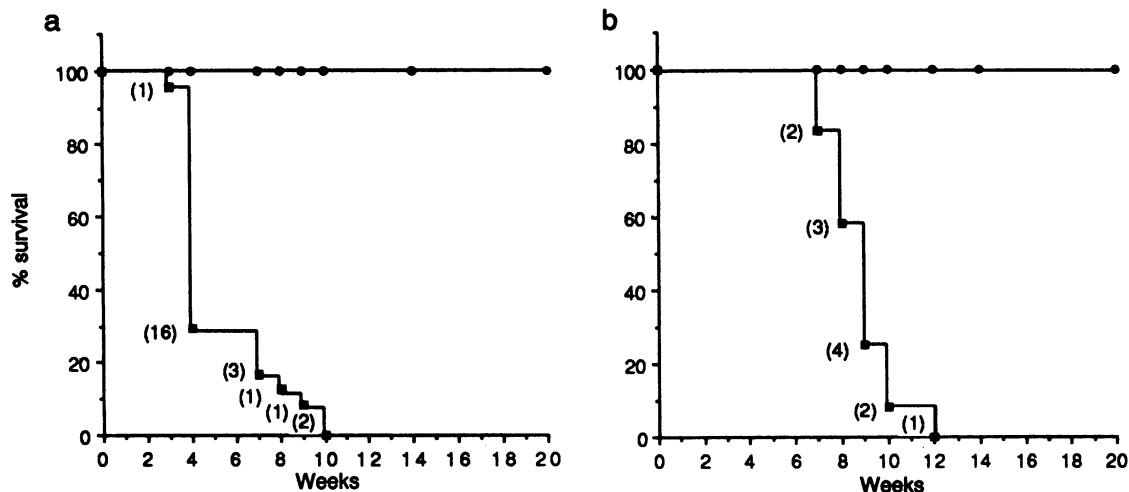


FIG. 1. Survival curves of  $\beta_2m^{-/-}$  (■) and C57BL/6 (●) mice infected with *M. tuberculosis* (24 mice per group) (a) or immunized with BCG and challenged with *M. tuberculosis* i.v. (12 mice per group) (b). Number of moribund or dead mice is indicated in parentheses beside each point.

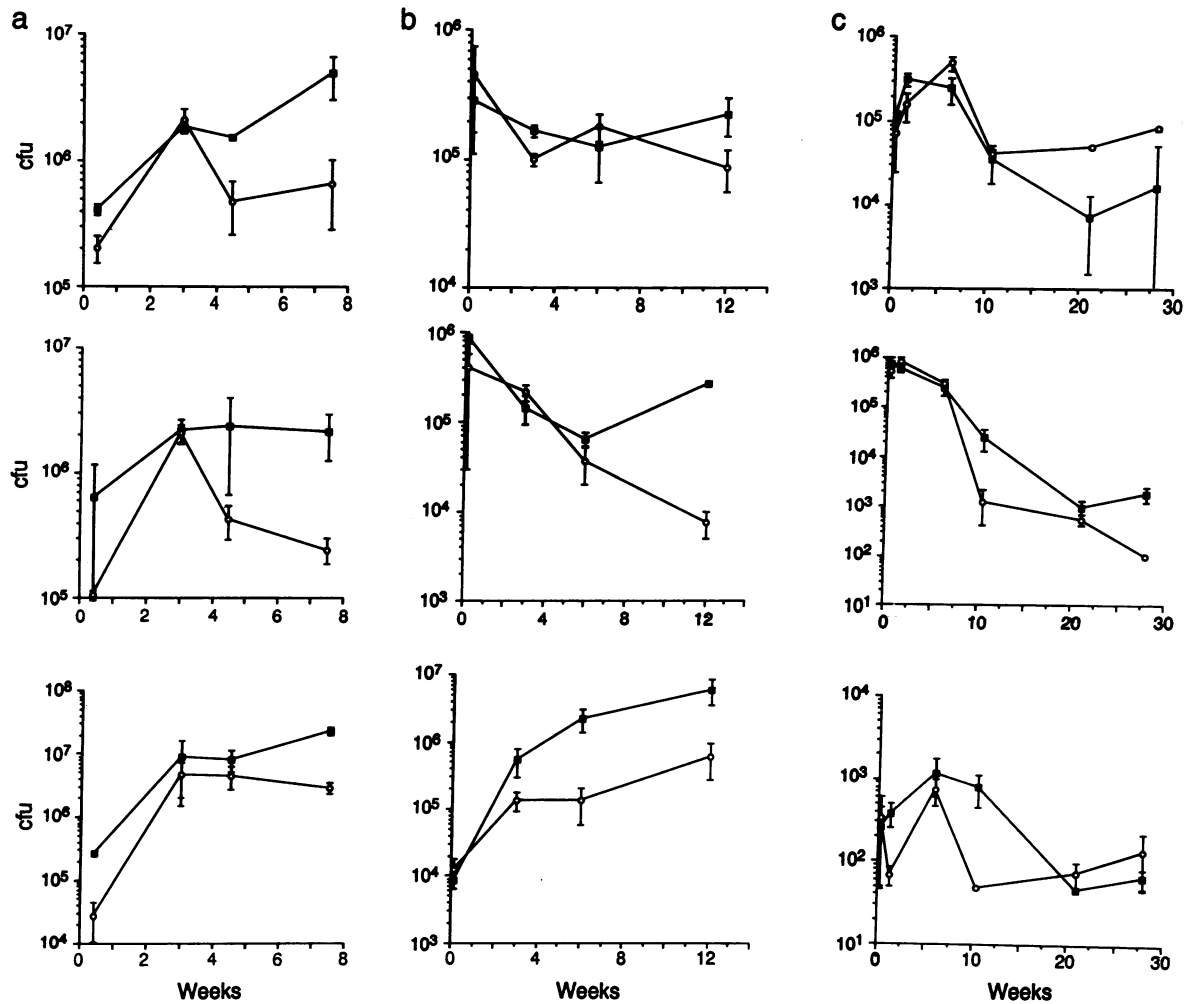


FIG. 2. Bacterial load (cfu) per organ [spleen (Top), liver (Middle), and lungs (Bottom)] in  $\beta_2m^{-/-}$  (■) and C57BL/6 (○) mice after *M. tuberculosis* challenge (a), immunization with BCG and challenge with *M. tuberculosis* (b), or infection with BCG (c). Four mice per time point were sacrificed. Experiments were repeated at least twice.

production in cells from C57BL/6 mice relative to  $\beta_2m^{-/-}$  cells (Table 1). Neither cytokine was produced in unstimulated spleen cells, nor did cells from uninfected mice respond to PPD or *M. tuberculosis* sonicate. Although not all infected mice had detectable cytokines in their sera, average IFN- $\gamma$  levels in sera from  $\beta_2m^{-/-}$  were slightly lower than in sera from C57BL/6 mice, while serum levels of TNF- $\alpha$  did not differ between the two mouse strains. Thus the qualitative differences in the course of infection in  $\beta_2m^{-/-}$  and control mice cannot be explained by different levels of production of these two important cytokines.

## DISCUSSION

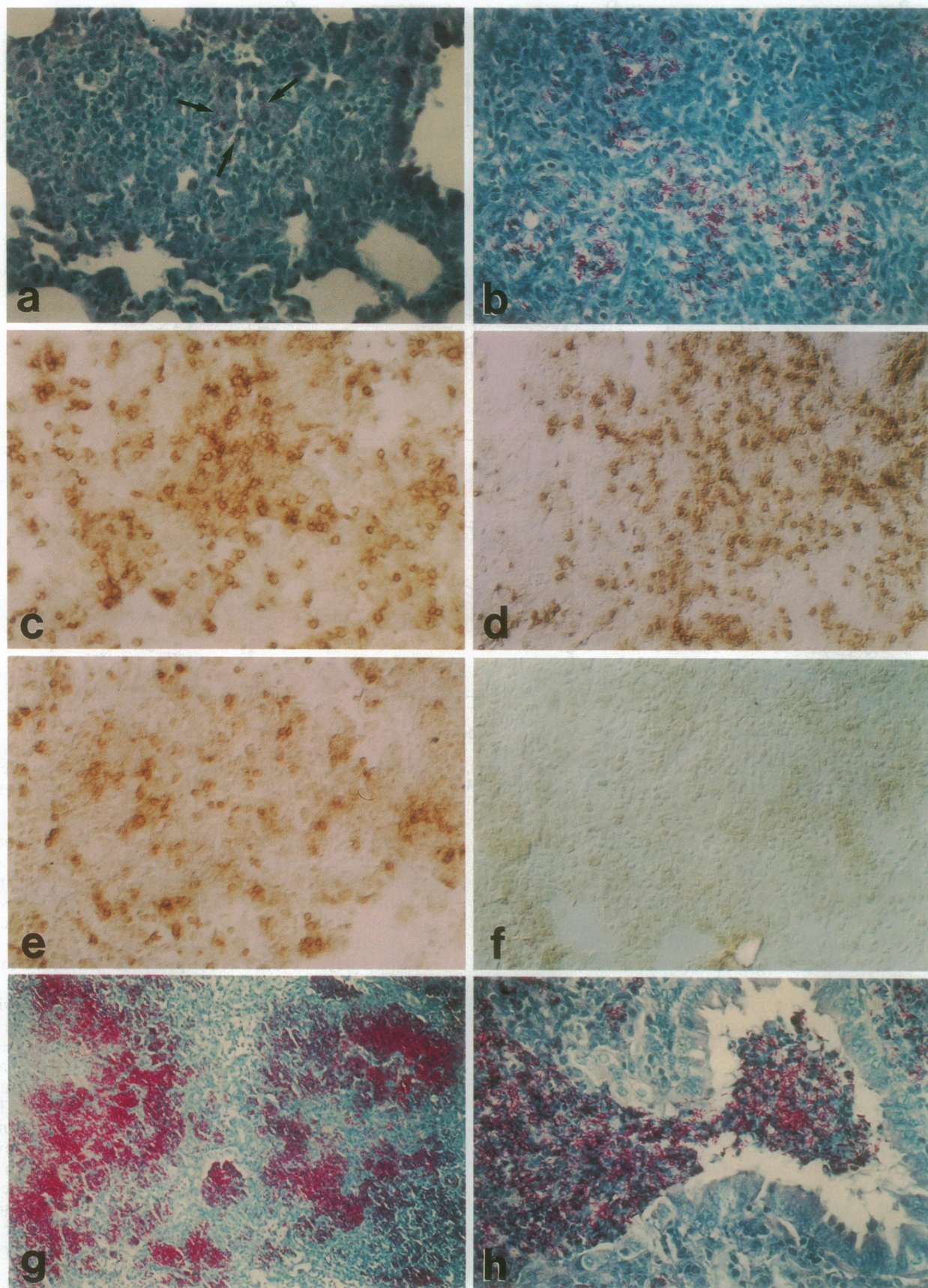
Defining the immune responses necessary and sufficient for protection against *M. tuberculosis* infection is an urgent scientific problem. All studies suggest that CD4 T cells play a vital role in the response to this pathogen (4–6). Cytokines, such as TNF- $\alpha$  and IFN- $\gamma$ , are also important in the control and pathology of *M. tuberculosis* infection (13, 14). However, the importance of CD8 T cells in protection and pathogenesis in tuberculosis has been problematic. Adoptive transfer and *in vivo* T-cell subset depletion studies have suggested that CD8 T cells (8), CD4 T cells (5, 6, 15), or both (7, 9, 16) are involved in controlling *M. tuberculosis* infection. *In vitro* studies indicate that murine CD8 T-cell lines from mice infected with mycobacteria are cytolytic for macrophages infected with live or dead mycobacteria (17, 18), yet their

ability to provide protection *in vivo* against *M. tuberculosis* challenge has not been established. We chose a genetic model in which mice fail to express MHC class I molecules on their cell surface, and therefore lack CD8 T cells, to assess the importance of MHC class I-restricted T cells to protection from experimental *M. tuberculosis* infection.

In this study, we observed a profound difference in the course of infection with *M. tuberculosis* between the  $\beta_2m^{-/-}$  mice and control mice. The control mice survived the *M. tuberculosis* challenge for >20 weeks, while the majority of  $\beta_2m^{-/-}$  mice did not survive beyond 6 weeks. BCG immunization provided some initial protection against a virulent *M. tuberculosis* challenge in  $\beta_2m^{-/-}$  mice, as reflected in an increased survival time of 3–4 weeks. There was a reduction in bacterial load in control and  $\beta_2m^{-/-}$  mice immunized with BCG, but this was not sufficient to prevent death in  $\beta_2m^{-/-}$  mice. Thus, a major difference in the response of  $\beta_2m^{-/-}$  mice to a tuberculosis infection, compared with either parent (C57BL/6 or 129) strain, was seen, indicating that the  $\beta_2m^{-/-}$  defect is responsible for the decreased survival and increased pathology in *M. tuberculosis*-infected mutant mice.

Although the  $\beta_2m^{-/-}$  mutation caused a significant reduction in the ability of the mice to control a virulent tuberculosis infection, the lack of class I MHC expression did not compromise the ability to control BCG infection. This points to an important difference between BCG and virulent *M. tuberculosis* strains in terms of their interaction with the host.





**FIG. 3.** Histology and immunohistochemistry of lung sections from C57BL/6 (*a*, *c*, and *e*) and  $\beta_2m^{-/-}$  (*b*, *d*, *f*, *g*, and *h*) mice. (*a* and *b*) Ziehl-Neelsen staining for AFB in granuloma; arrows indicate AFB. ( $\times 250$ .) (*c* and *d*) Immunohistochemistry with rat anti-mouse CD4. ( $\times 250$ .) (*e* and *f*) Immunohistochemistry with rat anti-mouse CD8. ( $\times 250$ .) (*g*) Ziehl-Neelsen staining of necrotizing granuloma in  $\beta_2m^{-/-}$  lung section. ( $\times 63$ .) (*h*) Ziehl-Neelsen staining of  $\beta_2m^{-/-}$  lung section showing tuberculous bronchopneumonia. ( $\times 250$ .)



Table 1. Cytokine production in spleen cells from C57BL/6 and  $\beta_2m^{-/-}$  mice

Mice	IFN- $\gamma$ , $\mu\text{g/ml}$			TNF- $\alpha$ , pg/ml		
	Con A	PPD	Sonicate	Con A	PPD	Sonicate
C57BL/6	31.7 $\pm$ 10.2*	15.7 $\pm$ 4.7**	16.1 $\pm$ 5.2*	235.0 $\pm$ 48.0*	201.7 $\pm$ 25.9*	476.7 $\pm$ 50.9*
$\beta_2m^{-/-}$	13.7 $\pm$ 5.6	004.5 $\pm$ 0.9	8.2 $\pm$ 2.4	191.7 $\pm$ 46.4	86.7 $\pm$ 13.3	600 $\pm$ 47.3

Spleen cells were stimulated with Con A, PPD, or *M. tuberculosis* sonicate. \*,  $P \geq 0.05$ ; \*\*,  $P = 0.02$ .

Histopathological examination of  $\beta_2m^{-/-}$  lungs showed that comparable granuloma formation occurred following either BCG immunization or tuberculosis infection. Most AFB were contained inside granulomas, but the replication of *M. tuberculosis* in the  $\beta_2m^{-/-}$  granulomas was uncontrolled. Most strikingly, only  $\beta_2m^{-/-}$  mice had extensive caseating necrosis of the lung tissue, characterized by numerous necrotizing granulomas and large numbers of AFB, many of which appeared to be extracellular amidst necrotic cell debris. Necrosis was not seen in control (C57BL/6 or 129) mice. The presence of necrosis appeared to correlate inversely with survival of the mouse.

An intrinsic limitation of the  $\beta_2m^{-/-}$  mouse model is that it is not possible to reconstitute the defect by adding back CD8 T cells to the mice; the lack of functional class I MHC molecules precludes presentation by potential targets, even to presensitized CD8 T cells, and thus prevents the expansion or functioning of repopulating cells. While CD8 CTLs have not been found in the  $\beta_2m^{-/-}$  mice (19, 20), it is not possible in the present model to establish formally that the lack of class I-restricted CD8 CTLs is responsible for the increase in mortality in a *M. tuberculosis* infection in these mice. Since there is no known defect in MHC class II-restricted CTLs (19, 20) or lymphokine production (21) in  $\beta_2m^{-/-}$  mice, our results indicate that CD4 T cells are not sufficient to control *M. tuberculosis* infection in mice. Natural killer cells are also present in  $\beta_2m^{-/-}$  mice (20, 22) and have been found to increase markedly in  $\beta_2m^{-/-}$  mice infected with *Toxoplasma gondii* (F. Y. Denkers, R. T. Gazzinelli, and A. Sher, personal communication). We challenged beige mice, defective in natural killer cell function, with *M. tuberculosis* and observed no significant differences in survival between C57BL/6 *bg/bg* and C57BL/6 control mice, up to 20 weeks (J.L.F. and K.J.T., unpublished data).

The most obvious interpretation of the present results is that CD8 T cells are functioning as MHC class I-restricted CTLs in a *M. tuberculosis* infection, and the absence of CTLs results in an increase in bacterial load and pathology in the mouse. As Kaufmann (18) has suggested from *in vitro* data, CTLs may be functioning in bacterial infections *in vivo* to lyse highly infected macrophages and disperse the bacteria to macrophages that can be activated. Some  $\gamma\delta$  T cells have been shown to recognize mycobacterial antigens (23), and also MHC class Ia antigens, such as TL and Qa1 (24), which may require  $\beta_2m$  for expression, but their recognition of antigen has not been reported to be MHC class I-restricted. Nevertheless, the possibility that their activity is diminished in  $\beta_2m^{-/-}$  mice cannot be excluded. Similarly, the existence of a subset of CD8 T cells producing cytokines uniquely necessary for the activation of macrophages cannot be excluded on the basis of present results. However, dramatic differences in IFN- $\gamma$  and TNF- $\alpha$  levels between the  $\beta_2m^{-/-}$  and control mice were not seen.

The present results establish that MHC class I-restricted T cells are necessary for protection against a virulent *M. tuberculosis* infection in mice. They raise more general questions, including whether BCG is a useful challenge

model for *M. tuberculosis* vaccine studies and whether BCG vaccines can effectively engender the functional CD8 or  $\gamma\delta$  T cells required for protection against *M. tuberculosis*. The results also emphasize the need to define the antigen specificity of cytotoxic CD8 T cells in tuberculosis, and suggest that new vaccines may be developed that are more effective in generating the appropriate protective T-cell response.

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