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Perforin, a Cytotoxic Molecule Which Mediates Cell Necrosis, Is Not Required for the Early Control of Mycobacterial Infection in Mice

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Host defense against mycobacterial infection requires the participation of monocytes and T cells. Both $CD4^+$ and $CD8^+$ T cells have been shown to be important in resistance to mycobacterial infection in vivo. The main contribution of $CD4^+$ T cells to the protective antituberculosis response involves the production of Th1-type cytokines, including interleukin-2 (IL-2) and gamma interferon (IFN-γ). $CD8^+$ T cells have been considered to be responsible primarily for cytotoxicity mediated by toxic molecules, including perforin. $CD8^+$ T cells may also elaborate Th1-type cytokines, such as IFN-γ, in response to the infection. To elucidate the contribution of perforin-mediated target cell death to the control of mycobacterial infection in vivo, mice with a disruption in the perforin gene ($P^{-/-}$) were infected with *Mycobacterium bovis* BCG or *M. tuberculosis* Erdman for 5 and 13 weeks, respectively. At 1, 3, 5, and 13 weeks postinfection, the number of viable mycobacteria in the lungs, spleens, and livers of mice were determined by CFU assay. The infected tissues were examined histologically, and cytokine mRNA levels in the spleens of these mice were determined. Similar studies were carried out in Fas receptor-defective (CBA/lpr^{cg}) mice to evaluate the contribution of this alternative cytotoxic pathway to the control of mycobacterial infection. The absence of either perforin gene function or Fas receptor gene function did not modify the course of experimental mycobacterial infection in these mice. In addition, both $P^{-/-}$ and Fas receptor-defective mice appeared to have a compensatory activation of cytokine genes, even in the absence of the experimental infection. $P^{-/-}$ mice had a mean 3.4- to 5-fold increase in mRNA levels for IL-10, IL-12p35, IL-6, and IFN-γ. Similarly, Fas receptor-defective mice had a mean 3- to 3.6-fold increase in mRNA levels for IFN-γ, IL-12p35, and IL-10. Our results indicate that both perforin-mediated cytotoxicity and Fas-mediated cytotoxicity do not appear to be necessary for the early control of mycobacterial infection in vivo.

$CD4^+$, as well as $CD8^+$, T cells have been shown to be important in resistance to mycobacterial infection in vivo (2, 12). However, the precise role of each of these cells in the control of the infection has not been fully elucidated. The main contribution of $CD4^+$ T cells to the protective antituberculosis response may involve the production of Th1-type cytokines, including interleukin-2 (IL-2) and gamma interferon (IFN-γ). These cells have also been shown to have some cytotoxic activity against mycobacterium-pulsed and mycobacterium-infected macrophages (1, 14). $CD8^+$ T cells are also capable of producing cytokines, including IFN-γ (13), but have been considered to be primarily responsible for cell-mediated cytotoxicity. $CD8^+$ T cells exert their killing effect by releasing cytoplasmic granules which contain a number of toxic molecules, including perforin (8). Upon contact with an antigen-presenting target cell, degranulation occurs and perforin is released. Once released, perforin inserts itself into the target cell membrane, aggregates, and causes the formation of pores. This results in osmotic disregulation and target cell death by a necrotic process (8). In addition, $CD8^+$ T cells can mediate cytotoxic effects via the interaction between Fas ligands on the T-cell surface and Fas receptors expressed on the target cells (11, 15, 18).

Perforin-mediated cytotoxicity has been implicated in in vivo clearance of some intracellular bacteria and virus. For instance, mice with a targeted disruption in the perforin gene ($P^{-/-}$ mice) infected with *Listeria monocytogenes* showed delayed clearance of the organisms in both primary and secondary infections compared with control mice (4). Also, clearance of a noncytopathic lymphocytic choriomeningitis virus is mediated through the perforin-dependent mechanism without any measurable involvement of the Fas ligand-Fas receptor pathway (5).

Previously, Flynn et. al showed that mice with a disruption in the β2 microglobulin gene failed to develop functional $CD8^+$ T cells and succumbed to virulent *Mycobacterium tuberculosis* infection much more rapidly than did control mice (2). Those investigators interpreted these findings to indicate that major histocompatibility complex class I-restricted ($CD8^+$) T cells may be required for resistance to *M. tuberculosis* infection in vivo. However, how $CD8^+$ T cells contribute to the control of mycobacterial infection is not fully understood.

To determine whether perforin-mediated cytotoxicity plays a role in the early control of mycobacterial infection, $P^{-/-}$ mice were infected intravenously with *M. bovis* BCG (a nonvirulent organism) or *M. tuberculosis* Erdman (an organism virulent in mice). At 1, 3, 5, and 13 weeks postinfection, we determined the numbers of viable mycobacteria in the lungs, spleens, and livers of mice by CFU assay, examined the infected tissues histologically, and measured cytokine mRNA levels in the spleens of these mice. Since cytotoxic T cells from $P^{-/-}$ mice have been shown to retain the Fas lytic pathway (6, 9, 19),

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similar studies were carried out with Fas receptor-defective mice to evaluate the contribution of this alternative killing pathway to the control of mycobacterial infection.

MATERIALS AND METHODS

Mice. The original perforin-deficient ($P^{-/-}$) mice were 129/C57BL/6 *H-2*^{b}/*H-2*^{b} (19). At the Rockefeller University Animal Facility, these were backcrossed to C57BL/6 mice to obtain a more homogenous genetic background. Control littermates were also from our breeding facilities; some additional control C57BL/6 animals were purchased from Jackson Laboratory (Bar Harbor, Maine). CBA/lpr^{cg} *H-2*^{k} Fas receptor-defective mice and control littermates ($CBA^{+/+}$) were kindly provided by Keith B. Elkon. CBA/lpr^{cg} mice have a point mutation in the receptor that renders it nonfunctional. All mice were 2 to 3 months old at the beginning of the study.

Mycobacteria. *M. bovis* bacillus Calmette-Guerin (BCG) Pasteur strain 1011 (Trudeau Institute, Saranac Lake, N.Y.) was grown in lipopolysaccharide-free Proskauer-Beck medium containing 0.05% Tween 80. Aliquots were stored in liquid nitrogen and, when thawed, shown to contain $\sim 2 \times 10^8$ CFU/ml and to be capable of growing on 7H10 solid medium. *M. tuberculosis* Erdman was provided by the Trudeau Institute mycobacterial culture collection.

Experimental design. Control littermates and mice with a disruption in the perforin gene ($P^{-/-}$) were injected intravenously in a lateral tail vein with 4×10^5 BCG or 2×10^5 *M. tuberculosis* Erdman organisms per mouse. Control mice and Fas receptor-defective (CBA/lpr^{cg}) mice were injected intravenously with 4×10^5 BCG organisms per mouse. At 1, 3, 5, and 13 weeks postinfection, the infected $P^{-/-}$ mice, Fas receptor-defective mice, and control littermates were sacrificed. Portions of infected organs were fixed in 10% formalin (Fisher Scientific), sectioned, and examined histologically. Viable mycobacteria from portions of infected tissues were determined by CFU assay. In addition, portions of spleens of both uninfected and infected mice were collected and immediately frozen at −70°C for determination of cytokine mRNA levels by reverse transcription (RT)-PCR.

CFU assay. Bacterial loads in the lungs, livers, and spleens of infected mice were evaluated by plating 10-fold serial dilutions of organ homogenates in phosphate-buffered saline onto Middlebrook 7H10 agar plates (Difco Laboratories, Detroit, Mich.). Plates were incubated at 37°C for 2 to 3 weeks. Bacterial colonies were counted with a dissecting microscope. For each culture, six replicate samples were counted and the mean was calculated.

Histologic evaluation. Organs were fixed in 10% buffered formalin for 24 h, paraffin embedded, and processed for histology. Sections were stained with hematoxylin-eosin for histologic examination and with Ziehl-Neelsen stain for evaluation of acid-fast bacilli by light microscopy. To determine the relative numbers of granulomata in infected $P^{-/-}$ mice, Fas receptor-defective mice, and control littermates, 10 random low-magnification microscopic fields of infected livers were examined and the granulomata were counted.

In situ staining of apoptotic cells. Organs were fixed in formalin overnight and paraffin embedded, and 6-μm-thick sections were placed on silanized slides (Oncor, Gaithersburg, Md.). The staining procedures described by the manufacturer (Oncor) for the ApopTag Peroxidase kit were modified as follows: the anti-digoxigenin-peroxidase was diluted 1:3 (vol/vol) in phosphate-buffered saline and stained with a diaminobenzidine substrate solution containing hydrogen peroxide for 1 min.

RT-PCR. Total RNA was prepared from the spleens of both uninfected and infected mice by using the RNAzol B solution (Tel-Test, Inc., Friendswood, Tex.) in accordance with the manufacturer's instructions. cDNA was synthesized from 1 μg of the isolated RNA by using Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Norwalk, Conn.). PCR was performed with the paired 5′ and 3′ primers for a specific cytokine by the following protocol: 1 cycle of 3 min at 94°C, 1 min at 55°C, and 2 min at 72°C and 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A negative control reaction in which no RNA template was added was included in each experiment. In each PCR, a trace amount of [^{32}P]dCTP (2×10^5 cpm/mixture) was included to label the PCR product. The PCR products were subjected to electrophoreses on 1% agarose gels containing ethidium bromide and visualized by UV illumination. Following photodocumentation, the agarose gels were fixed in 10% trichloroacetic acid for 15 min at room temperature, washed twice with H_2O to remove the trichloroacetic acid, and dried under vacuum. The dried gels were exposed to XAR-5 autoradiographic films (Kodak). Cytokine mRNAs were quantitated by using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and results were first normalized to the amount of β-actin mRNA. The results are expressed as a fold increase over the baseline amount of cytokine message expressed in the uninfected control tissues.

RESULTS AND DISCUSSION

Mycobacterial infection of $P^{-/-}$ mice. $P^{-/-}$ mice and control ($P^{+/+}$) littermates were infected intravenously with either BCG or *M. tuberculosis* Erdman. Following BCG infection, there was no significant increase in the numbers of viable organisms in the lungs, spleens, or livers of the control mice during the course of infection (Fig. 1). The number of CFU recovered from the lungs of $P^{-/-}$ mice at 1 week postinfection was almost 1 log lower than that of the CFU obtained from the lungs of control littermates (Fig. 1). At 3 and 5 weeks postinfection, the number of CFU from infected lungs was also lower in $P^{-/-}$ mice; however, the difference was less pronounced. In the infected livers and spleens, the numbers of CFU of BCG obtained from $P^{-/-}$ mice were the same as those obtained from control mice at 1, 3, and 5 weeks postinfection. A slight decrease in the numbers of CFU was observed in both infected spleens and livers of all mice at 5 weeks postinfection.

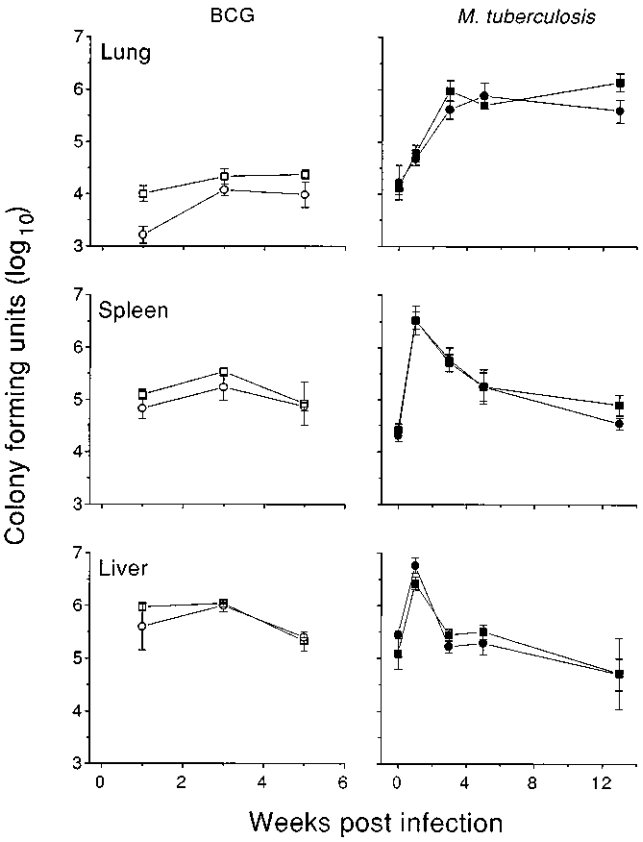

FIG. 1. Mycobacterial load expressed as CFU per organ in $P^{-/-}$ mice (circles) and control $P^{+/+}$ mice (squares). Mice were infected intravenously with either BCG (open symbols) or *M. tuberculosis* Erdman (closed symbols). Three to five mice per time point were sacrificed for CFU analysis. Results are means ± standard deviations of two experiments.

Unlike BCG infection, *M. tuberculosis* infection of $P^{-/-}$ and control mice resulted in a significant increase in the numbers of CFU in the infected lungs by 1 and 3 weeks postinfection. In infected spleens and livers, there was an initial increase followed by a decrease in the numbers of CFU (Fig. 1). The numbers of viable mycobacteria recovered from infected organs of $P^{-/-}$ and control mice were not significantly different in the two strains of mice at all of the time points and in all of the tissues studied. These findings suggest that *M. tuberculosis* was better able to replicate than BCG in the lungs of both $P^{-/-}$ and control mice. Furthermore, $P^{-/-}$ mice were as capable of controlling the infection by BCG and *M. tuberculosis* Erdman as the control littermates as evaluated by CFU assay (Fig. 1). These results indicate that perforin does not appear to play a

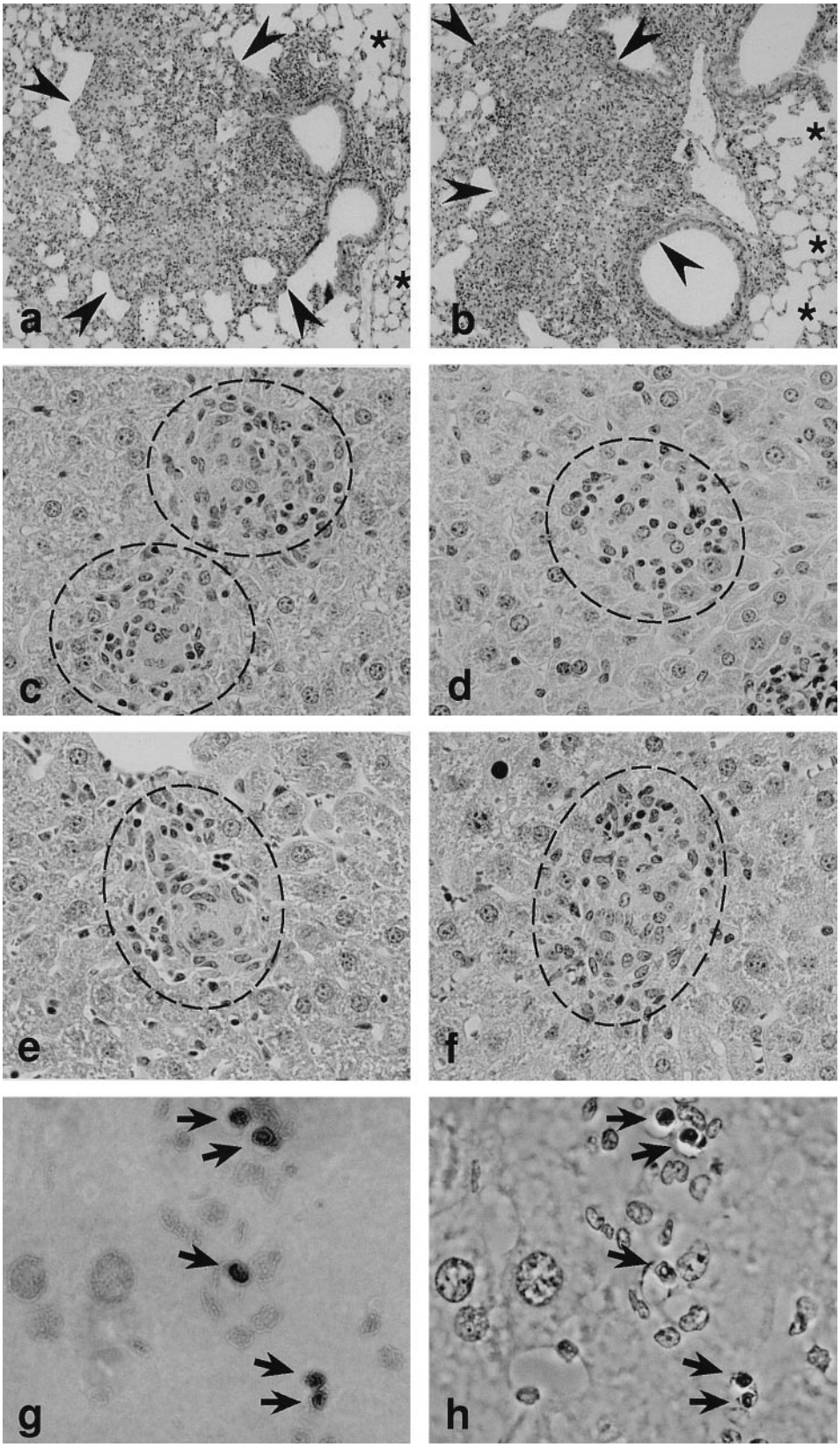

FIG. 2. Histology of mycobacterium-infected tissues. Lungs of $P^{-/-}$ (a) and control $P^{+/+}$ (b) mice at 3 weeks post *M. tuberculosis* Erdman infection are shown. Arrowheads demarcate the granulomata, which are surrounded by normal lung tissue (asterisks). Livers of $P^{-/-}$ (c) and control $P^{+/+}$ (d) mice at 3 weeks post BCG infection are shown. Granulomata are enclosed within the ellipses. Livers of Fas receptor-deficient CBA/lpr^{cg} (e) and control $CBA^{+/+}$ (f) mice at 3 weeks post BCG infection are shown. Granulomata are enclosed within the ellipses. Staining of apoptotic cells in the granulomata in the livers of Fas receptor-deficient CBA/lpr^{cg} mice infected with BCG (week 3) is shown in bright-field (g) and phase-contrast (h) images. Apoptotic cells (arrows) were stained with ApopTag Peroxidase as described in Materials and Methods and are visible as condensed phagocytosed nuclei. Magnifications: a and b, ×25; c to f, ×100; g and h, ×250.

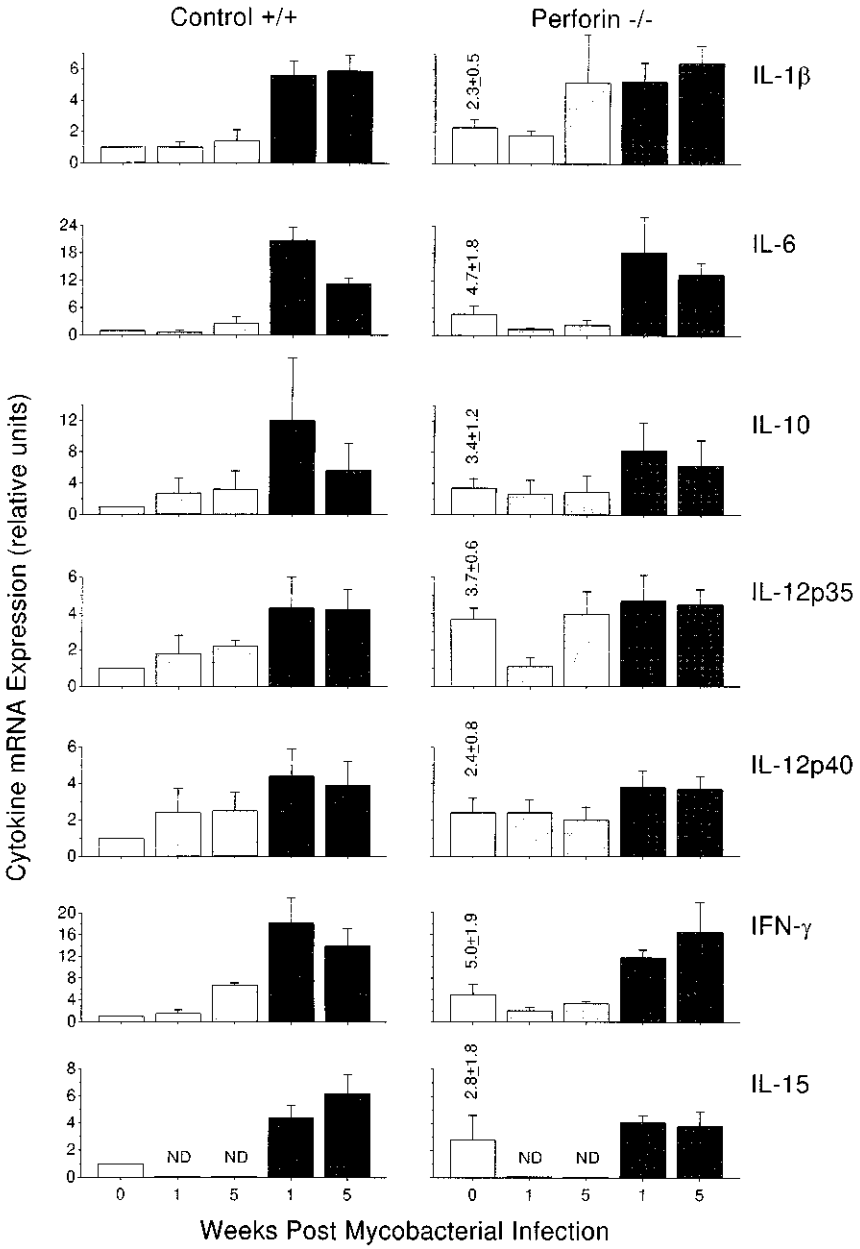

FIG. 3. Cytokine mRNA levels in the spleens of mice infected with BCG (light gray bars) or *M. tuberculosis* Erdman (dark gray bars). Spleens obtained from uninfected mice (open bars) and infected mice (shaded bars) were assayed by RT-PCR at 0, 1, and 5 weeks postinfection. Cytokine mRNA levels were quantitated as described in Materials and Methods. Results were first normalized to the amount of β-actin mRNA in each lane and then expressed as the fold increase over the amount of cytokine message expressed by uninfected $P^{+/+}$ control mice at time zero. ND, not done. The values above the time zero cytokine levels of $P^{-/-}$ mice are the fold increase over time zero cytokine levels of control $P^{+/+}$ mice normalized to 1. The results are means ± standard errors of the means for the three infected and six uninfected mice used per test group from two experiments.

protective role in the early control of either avirulent (BCG) or virulent (*M. tuberculosis*) mycobacterial infection.

Histologic evaluation of infected organs. The lungs and livers of both $P^{-/-}$ and control mice infected with either BCG or *M. tuberculosis* were examined. No significant morphologic difference was observed between $P^{-/-}$ and control mice at any time point (Fig. 2). In either $P^{-/-}$ or control mice infected with BCG, granulomata were rarely seen in the lungs. In contrast, the lungs of both $P^{-/-}$ and control mice infected with *M. tuberculosis* contained many large granulomata (Fig. 2a and b, arrowheads) surrounded by normal lung tissue (Fig. 2a and b, asterisks). However, in the livers of control mice, both BCG and *M. tuberculosis* infections resulted in granuloma formation as early as 1 week postinfection (Fig. 2d). The same morphology was observed in $P^{-/-}$ livers following either infection (Fig. 2c). Thus, granuloma formation induced in response to mycobacterial infection was not affected in either lungs or livers by the disruption in the perforin gene in $P^{-/-}$ mice. Together with the CFU findings, these results suggest that the absence of an intact perforin gene did not render $P^{-/-}$ mice more susceptible to either avirulent or virulent mycobacterial infection.

TABLE 1. CFU per organ in Fas receptor-deficient CBA/lpr^{cg} and control CBA$^{+/+}$ mice infected with BCG

Infected tissue and mouse	Log_{10} CFU[a] at postinfection wk:		
	1	3	5
Lung			
CBA/lpr^{cg}	3.44 ± 0.30	4.64 ± 0.06	4.38 ± 0.07
CBA$^{+/+}$	3.62 ± 0.97	4.66 ± 0.05	4.56 ± 0.21
Liver			
CBA/lpr^{cg}	5.84 ± 0.07	5.59 ± 0.19	5.39 ± 0.59
CBA$^{+/+}$	5.91 ± 0.07	5.59 ± 0.57	5.39 ± 0.80
Spleen			
CBA/lpr^{cg}	4.96 ± 0.21	4.80 ± 0.73	4.10 ± 0.70
CBA$^{+/+}$	5.13 ± 0.13	5.44 ± 0.82	4.57 ± 0.04

[a] Results are means ± standard deviations from two separate experiments with two or three mice per group.

Systemic cytokine activation in P$^{-/-}$ mice. To better understand the immune response to mycobacterial infection in the two strains of mice, we investigated systemic leukocyte cytokine activation. RT-PCR analysis of cytokine mRNA levels in the spleen revealed that even at baseline before infection, P$^{-/-}$ mice had 3.4 to 5 times higher levels of IL-10 mRNA, IL-12p35 subunit mRNA, IL-6 mRNA, and IFN-γ mRNA in their spleens than did the uninfected control littermates (Fig. 3). Baseline (week 0) levels of the IL-12p40 subunit and IL-1β were higher (although not statistically significantly higher) in P$^{-/-}$ mice. Baseline levels of IL-15 mRNA were similar in both types of mice. Thus, it appeared that perforin gene disruption was associated with a compensatory immunologic activation of leukocytes and the expression of increased levels of leukocyte cytokine mRNAs, even in the absence of experimental infection.

Following both BCG and *M. tuberculosis* infections, cytokine genes were upregulated in both control and P$^{-/-}$ mice. There were no significant differences in cytokine levels between control and P$^{-/-}$ mice by 5 weeks postmycobacterial infection (Fig. 3). Thus, activation of cytokine genes in response to mycobacterial infection occurs in both control and knockout mice, blurring the baseline cytokine activation observed in the uninfected gene-disrupted mice. However, the virulent *M. tuberculosis* infection induced a response of greater magnitude.

Mycobacterial infection of Fas receptor-defective mice. Since it has been shown that cytotoxic T cells derived from perforin knockout mice retain the Fas-mediated cytotoxic pathway, it is possible that Fas-mediated killing is involved in the control of mycobacterial infection in vivo. Fas receptor-defective (CBA/lpr^{cg}) mice and control (CBA$^{+/+}$) mice were therefore infected with BCG. Similar to perforin-defective mice, Fas receptor-defective mice were capable of limiting BCG infection as effectively as the controls (Table 1). Granuloma formation in response to infection with BCG was rarely observed in the lungs of both types of mice. However, both Fas receptor-defective (Fig. 2e) and control (Fig. 2f) mice infected with BCG demonstrated granuloma formation in the liver as early as 1 week postinfection. The number and morphology (Fig. 2e and f) of the granulomata were similar for both strains of mice. Despite significantly reduced Fas receptor activity, apoptosis of leukocytes in the granulomata in livers of Fas receptor-defective mice was similar to that observed in the control mice (Fig. 2g and h). This finding suggests that even in the absence of an intact Fas-induced pathway, an alternative apoptotic pathway(s) contributes to cell turnover in the granuloma.

Fas receptor-defective mice also had enhanced baseline cytokine mRNA levels. IL-6 mRNA was increased 15.8-fold ± 12.3-fold in the spleens of Fas receptor-defective mice compared with the controls; IL-10, IL-12p35, and IFN-γ mRNA levels were increased 3.56-fold ± 0.45-fold, 3.30-fold ± 0.71-fold, and 2.96-fold ± 0.31-fold, respectively. IL-1β and IL-12p40 mRNA level increases were similar in both strains of mice (1.54-fold ± 0.37-fold and 1.57-fold ± 0.89-fold, respectively [means ± standard errors of the means for four mice per group]). Taken together, these findings suggest that Fas-mediated cytotoxicity is not involved in the early protective immune response to BCG challenge in vivo.

We have previously shown that human lymphokine-activated killer cells, which have a perforin-mediated cytotoxic mechanism, were able to lyse BCG-infected human monocytes in vitro, although the viability of the BCG organisms was not affected (10). This earlier finding is consistent with our in vivo observations reported here; that is, perforin-mediated cytotoxicity neither kills mycobacteria directly nor is it necessary for the control of early BCG infection. Although granzymes (serine proteases) present within the granules containing perforin molecules are capable of inducing DNA fragmentation, several studies reported that this cytotoxicity was dependent on the presence of perforin at sublytic doses (3, 16, 17). Due to the absence of perforin in the perforin-deficient mice used in our study (19), the contribution of granzymes to the control of mycobacterial infection seems unlikely.

We have also shown that Fas-mediated killing of infected monocytes in vitro does not reduce the viability of the intracellular mycobacteria (7). Thus, our observations suggest that both perforin-mediated cytotoxicity and Fas-mediated cytotoxicity are not necessary for the early control of mycobacterial infection in vivo. Furthermore, P$^{-/-}$ and Fas receptor-defective mice appear to have a compensatory activation of cytokine genes. If cytotoxic cells are indeed necessary for the protective immune response to mycobacterial challenge in vivo, our results suggest that the response is mediated by alternative cytotoxic molecules and/or the production of protective cytokines.

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