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CD8$^+$ CTL from Lungs of *Mycobacterium tuberculosis*-Infected Mice Express Perforin In Vivo and Lyse Infected Macrophages[1]

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CD8$^+$ T lymphocytes have been implicated in the protective immune response against human and murine tuberculosis. However, the functional role that this cell subset plays during the resolution of infection remains controversial. In this study, we demonstrate the presence of *Mycobacterium tuberculosis*-specific CD8$^+$ CTL in the lungs and lung-draining lymph nodes of mice infected with *M. tuberculosis* via the aerosol or i.v. route. These cells expressed perforin in vivo and specifically recognized and lysed *M. tuberculosis*-infected macrophages in a perforin-dependent manner after a short period of in vitro restimulation. The efficiency of lysis of infected macrophages was dependent upon the time allowed for interaction between macrophage and *M. tuberculosis* bacilli. Recognition of infected targets by CD8$^+$ CTL was β_2-microglobulin and MHC class I dependent and was not CD1d restricted. The presented data indicate that CD8$^+$ T cells contribute to the protective immune response during *M. tuberculosis* infection by exerting cytotoxic function and lysing infected macrophages. *The Journal of Immunology*, 2000, 165: 353–363.

Establishment of a protective immune response during the course of *Mycobacterium tuberculosis* infection requires successful recruitment of T lymphocytes (1). CD4$^+$ T lymphocytes are crucial for the development of protective immunity during infection (2–4). There is evidence that a protective immune response to *M. tuberculosis* infection also includes a CD8$^+$ T cell component. The involvement of CD8$^+$ T cells in control of the infection was initially demonstrated in adoptive transfer experiments (1) and in vivo T cell depletion experiments (3). Mice deficient in CD8$^+$ T cells due to disruptions in the genes for β_2-microglobulin (β_2m)[3] or TAP1 were more susceptible to *M. tuberculosis* infection than wild-type mice, confirming the protective role of MHC class I-restricted CD8$^+$ T cells against tuberculosis (5, 6).

The role that CD8$^+$ T cells play in the protective immune response against *M. tuberculosis* is controversial. CD8$^+$ T cells may function as a source of type 1 cytokines such as IFN-γ and TNF-α, similar to CD4$^+$ T cells. It has also been suggested that the protective effect of CD8$^+$ T cells is dependent upon the ability to lyse infected macrophages (Mϕ) within the tissues. In *Chlamydia trachomatis* infection, another intracellular pathogen, CD8$^+$ T cells induced protection by IFN-γ production (7), while CD8$^+$ T cells mediated protection in a *Listeria monocytogenes* model in an IFN-γ-independent manner (8).

Cytokine production by Ag-specific CD8$^+$ T cells upon in vitro restimulation has been demonstrated in human and murine studies (9–14). Partial protection of athymic mice from *M. tuberculosis* infection by CD8$^+$ T cells required IFN-γ production (15), suggesting that cytokine secretion by these cells was induced in vivo. However, it is not clear whether CD8$^+$ T cells contribute significantly to the cytokine pool during the normal course of infection. We showed previously that CD8$^+$ T cells freshly harvested from infected lung were capable of IFN-γ and TNF- α production, although it appeared that only a small subset actively produced cytokine at the site of infection (14). Human CD8$^+$ T cells stimulated with live *M. tuberculosis* bacilli or mycobacterial Ags produced fewer cytokine molecules per cell than did CD4$^+$ T cells (12).

In tuberculosis studies, mycobacteria-specific cytolytic CD8$^+$ T cells have been generated in mice and isolated from humans (10, 16–23). In addition to lysis of infected Mϕ, human CD8$^+$ T cells can directly kill mycobacteria by granulysin, a granule-associated protein of CD8$^+$ T cells (24). However, the existence of CD8$^+$ CTL in mice infected with virulent *M. tuberculosis* that are capable of lysing Mϕ infected with live *M. tuberculosis* has not been demonstrated. Moreover, no studies have addressed the presence of CD8$^+$ CTL in *M. tuberculosis*-infected lungs. The participation of cytotoxic CD8$^+$ T cells in the protective immune response against *M. tuberculosis* has been questioned by the findings that deficiencies in perforin, granzyme B, or FAS-receptor molecules did not affect short-term survival of *M. tuberculosis*-infected mice (25, 26). Although this may be due to compensatory mechanisms since doubly deficient mice were not tested in those studies, these results demand a more thorough investigation of the function of CD8$^+$ T cells induced during *M. tuberculosis* infection.

In this study, we demonstrate that mycobacteria-specific CD8$^+$ CTL are present in the lungs and lung-draining lymph nodes of *M. tuberculosis*-infected mice. These CD8$^+$ T cells specifically lysed *M. tuberculosis*-infected Mϕ mice in a perforin-dependent manner. Recognition of mycobacterial Ags by the CTL was β_2m dependent, MHC class I dependent, and CD1 independent. We also detected expression of perforin by lymphocytes in the organs

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; DC, dendritic cell; Mϕ, macrophage; P$^{-/-}$, perforin deficient.

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of infected mice, further supporting a role for the cytotoxic function of CD8$^+$ T cells in the protective immune response against *M. tuberculosis*.

Materials and Methods

Mice

Eight- to 10-wk-old female C57BL/6 (The Jackson Laboratory, Bar Harbor, ME), BALB/c (The Jackson Laboratory), β_2m$^{-/-}$ (The Jackson Laboratory), MHC class II$^{-/-}$, and perforin-deficient (P$^{-/-}$) mice were used. MHC class II$^{-/-}$ and P$^{-/-}$ mice were bred in the pathogen-free facility at the University of Pittsburgh School of Medicine (Pittsburgh, PA). All mice were maintained in specific pathogen-free biosafety level 3 facilities. All experimental and animal handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh School of Medicine.

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 infection, 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 tail vein with 2×10^5 live bacilli in 100 μl or by aerosol with ~100 live bacilli, as determined by viable counts on 7H10 agar plates (Difco Laboratories, Detroit, MI). For aerosol infections, 10^7 CFU/ml were placed in nebulizer and mice were exposed for 20 min, followed by 5 min of air only, using an InTox aerosol unit (Albuquerque, NM).

Culture and infection of DC and Mϕ

Dendritic cells (DCs) and Mϕ were grown from murine bone marrow precursors and cultured for 5 days using methods previously described (14). For Mϕ infection, adherent cells were washed twice with ice-cold PBS (Life Technologies, Grand Island, NY) and infection media was added containing DMEM, 10% certified FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine (Life Technologies). For DC infection, nonadherent cells were harvested, adjusted to 0.5×10^6 cells/ml in DC media containing recombinant murine GM-CSF, and dispersed into 25-cm^2 culture flasks (Costar, Cambridge, MA) for infection.

For infection of APC, frozen aliquots were used to start cultures at a concentration of 2.5×10^6/ml in liquid medium (7H9 Middlebrook; Difco, Detroit, MI); bacteria were grown in 5% CO_2 at 37°C. Four- to six-day-old cultures were used to infect cells. Bacteria were washed, resuspended in DMEM medium (Life Technologies), and sonicated for 15 s before infection of cell cultures. Cells were infected for 16–18 h at multiplicity of infection of 3–5. Extracellular bacteria were separated from cells by low speed centrifugation (DCs) or by washing adherent cells twice with PBS. Cells were incubated for 10 min on ice, and harvested by forceful pipetting. In some experiments, infected and uninfected Mϕ were cultured in fresh medium for an additional 24 h (see text) before use in CTL assays. For DCs and Mϕ, the percentage of infection was estimated in each experiment by staining aliquots of cells by the Kinyoun method for acid-fast bacteria (Difco). Routinely, 40–55% of DCs and 60–85% of Mϕ were infected.

FACS analysis of cell surface markers

Lung and lymph node cells were obtained from mice infected for various periods of time by crushing the organs in cell strainers (Becton Dickinson Labware, Lincoln Park, NJ) to obtain single cell suspensions. RBC were lysed with NH_4Cl/Tris solution, and cells were washed twice. Cells were stained for cell surface markers using Abs against CD8 (anti-CD8 CyChrome Ab, clone 53-6.7), CD4 (anti-CD4 CyChrome Ab, clone H129.19), CD44 (anti-CD44 FITC Ab, clone IM7), CD45RB (anti-CD45RB FITC Ab, clone 16A), and CD69 (anti-CD69 FITC Ab, clone H1.2F3) in PBS containing 20% mouse serum, 0.1% BSA, and 0.1% sodium azide for 30 min at 4°C. All Abs were used at 0.2 μg/10^6 cells and obtained from PharMingen (San Diego, CA). Cells were fixed with 4% paraformaldehyde for 4–15 h and analyzed by FACS using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were gated on the lymphocyte population by size.

Intracellular staining

Single cell suspensions of lungs at various times postinfection were prepared, as described above. Staining for intracellular cytokines was performed as described previously (14). Briefly, cells were either stimulated with anti-CD3 (clone 145-2C11, 0.1 μg/ml) and anti-CD28 (clone 37.51, 1 μg/ml) Abs (PharMingen, San Diego, CA) or left unstimulated for 5–6 h in the presence of 3 μM monensin (Sigma, St. Louis, MO). At the end of stimulation period, cells were stained for CD4 and CD8, fixed, permeabilized, and stained for intracellular cytokines. Intracellular perforin staining was performed as previously described (27). Briefly, cells were fixed in 2% paraformaldehyde for 20 min on ice, washed, and permeabilized with 0.1% saponin. Cells were stained with anti-perforin Ab diluted 1/200 (clone KM 585; Kamiya Biomedical, Seattle, WA) in staining buffer containing 0.1% BSA, 0.1% sodium azide, 0.3% saponin, and 20% mouse serum at 4°C for 30 min, followed by staining with anti-rat IgG (anti-rat IgG2a FITC Ab, clone RG7/1.30; PharMingen). Cells were washed with 0.1% saponin, stained with anti-CD8 Ab or anti-CD4 Ab, and fixed in 4% paraformaldehyde for 1 h before analysis.

Culture of lung and lymph node cells

Lung and lymph node cells from mice infected for 2–5 wk were obtained as described above and plated in 96-well U-bottom plates (Corning, Corning, NY) in DMEM supplemented with 10% certified FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES (Life Technologies), 50 μM 2-ME (Sigma, St. Louis, MO), 30 μg/ml gentamicin (Life Technologies, Gaithersburg, MD), 15–20 U/ml recombinant murine IL-2 (Boehringer Mannheim, Indianapolis, IN), and 1 mM aminoguanidine (Sigma) at 2×10^5 cells/well. MHC class II$^{-/-}$ DCs infected for 18–24 h, as described above, were added to the cell cultures at $6.5–7 \times 10^3$ viable cells/well. After 2–3 days of culture, 100 μl of media was removed from each well and replaced with fresh media containing IL-2. Cells were cultured for additional 3–4 days before FACS analysis and CTL assays.

Perforin immunohistochemistry

Organs were fixed in 2% paraformaldehyde before paraffin embedment and sectioning. For immunohistochemistry, polyclonal anti-mouse perforin Ab was generated in rabbits, as described previously (28, 29). Sections were deparaffinized and stained using methods previously described (29). Briefly, sections were stained using rabbit anti-perforin Ab diluted 1/300, followed by biotinylated anti-rabbit IgG (Vector, Burlingame, CA). Perforin Ab was visualized using ABC kit (Vector) and 3-amino-9-ethyl-carbazole substrate (Sigma). Immunoreactivity was indicated by the appearance of the red-brown color.

Cytotoxicity assays

Lymphocytes harvested from 5–7-day stimulation cultures were tested in a 4-h ^{51}Cr release assay. Where indicated, 15 mM strontium chloride (Aldrich Chemical, Milwaukee, WI) was added to the T cell cultures for the final 12 h of culture. No cytotoxicity associated with strontium treatment was observed. To prepare targets, Mϕ uninfected or infected for indicated periods of time were harvested as described above, and $1–2 \times 10^6$ cells were labeled with 100 μl of $Na^{51}CrO_4$ (Amersham) in Teflon jars (Savillex, Minnetonka, MN) for 1 h at 37°C. Cells were washed three times with DMEM, added to wells of 96-well U-bottom plates (Corning, Corning, NY) at 4×10^3 cells/well, and allowed to adhere for 20 min before addition of T cells. Cultured cells were added at various E:T ratio in a total volume of 0.1 ml in DMEM supplemented with10% certified FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES, and 50 μM 2-ME, and assay was conducted for 4 h. Where indicated, targets were incubated for 2 h with increasing doses of perforin purified, as described previously (28). Where indicated, anti-MHC class I supernatant (anti-K^{b}D^b, clone 28-8-6, 1/2 dilution) or anti-CD1d Ab (clone 19G11, 20 μg/ml) was included in the CTL culture. After 2–4 h, 85 μl of supernatant was removed from each well without disturbing the cells and counted in gamma counter. Spontaneous release was determined by culturing target cells in medium alone, and total release was determined by adding 0.1% Triton-X to target cells. Percent of specific lysis was calculated by the formula: $100 \times$ [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)].

Statistics

The paired Student's test was used to compare groups. Statistical analysis was performed using StatView (Abacus Concepts, Berkeley, CA). A p value of ≤ 0.05 was considered significant.

Results

Culture and characterization of effector CD8$^+$ T cells from lungs and lung-draining lymph nodes of M. tuberculosis*-infected mice*

Lung-draining lymph nodes of mice infected i.v. with *M. tuberculosis* were examined for the presence of activated effector CD8$^+$

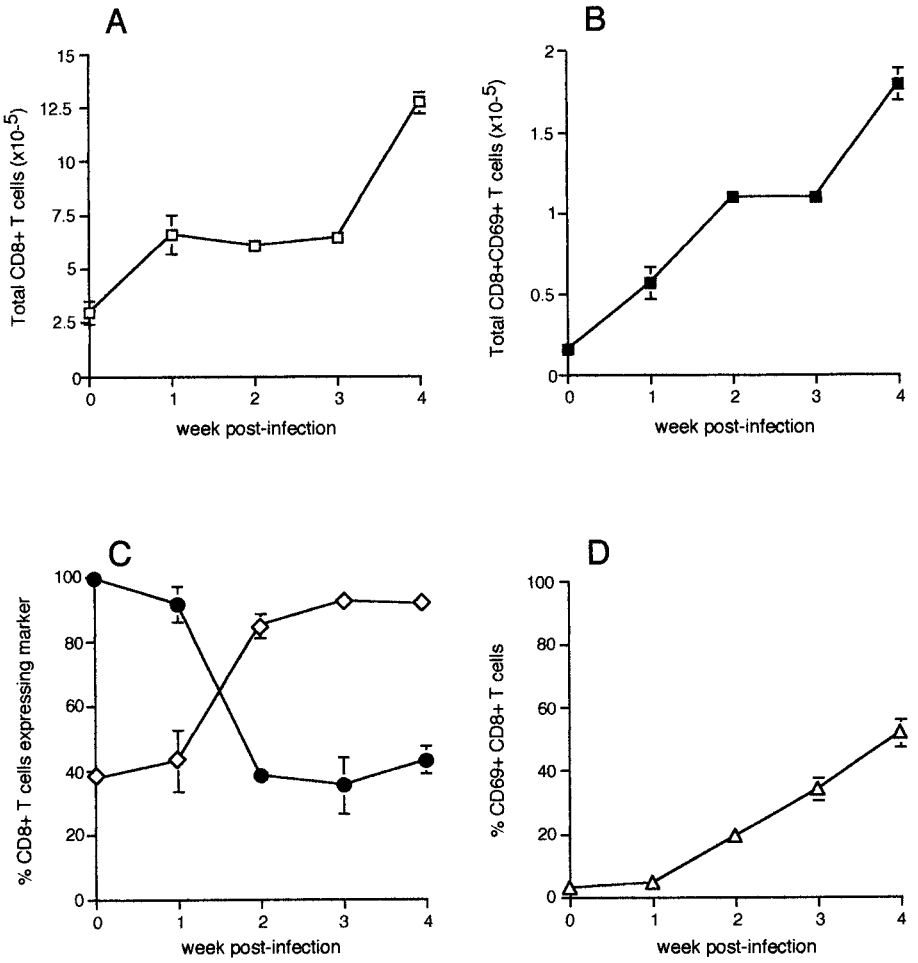

FIGURE 1. Changes in numbers and activation profiles of $CD8^+$ T cells in the lungs and lymph nodes following *M. tuberculosis* infection. C57BL/6 mice were infected with $1.5–2 \times 10^5$ viable *M. tuberculosis* bacilli (Erdman strain); lung-draining lymph nodes (*A* and *B*), and lungs (*C* and *D*) were harvested 0, 1, 2, 3, and 4 wk postinfection. *A*, Numbers of total viable cells in the lymph nodes were counted by trypan blue exclusion. The cells were stained for CD8, gated on lymphocyte population by size, and analyzed by flow cytometry. The obtained percentages of $CD8^+$ T cells were used to calculate total numbers of $CD8^+$ T cells. For each time point, $p < 0.05$ compared with uninfected control (time 0). *B*, Cells were harvested from lymph nodes, stained for CD8 and CD69, and analyzed by two-color flow cytometry. The numbers of $CD69^+$ cells were determined as in *A*. For each time point, $p < 0.05$ compared with uninfected control. *C*, Cells were harvested from lungs; stained for CD8, CD44, and CD45RB; and gated on lymphocytes. Cells were further gated on CD8, and expression of CD44 (open diamonds) and CD45RB (closed circles) in the gate was analyzed. For 2-, 3-, and 4-wk time points, $p < 0.05$ compared with uninfected control. *D*, Cells harvested from the lungs were stained for CD8 and CD69, and analyzed by two-color flow cytometry after gating on lymphocytes by size. For each time point, $p < 0.05$ compared with uninfected control. Each time point in *A–D* represents 3–10 mice. Error bars represent SE.

T cells. The changes in the T cell composition of lung-draining lymph nodes during the infection are summarized in Fig. 1, *A* and *B*. As early as 1 wk postinfection, the number of $CD8^+$ T cells in the lymph nodes of infected mice doubled (Fig. 1*A*), and the number of $CD69^+$ $CD8^+$ T cells increased 3–4-fold (Fig. 1*B*), indicating that $CD8^+$ T cells were recruited to the lymph nodes and were undergoing priming. CD69 expression is frequently used as an indication that T cells have encountered APC. This molecule is rapidly up-regulated upon triggering of TCR, reaching a peak at 24–48 h (30–32). By 4 wk postinfection, the total number of $CD8^+$ T cells in the lymph nodes was increased ~5-fold as compared with uninfected controls, and total numbers of $CD69^+$ $CD8^+$ T cells were increased ~10-fold (Fig. 1, *A* and *B*). However, a significant proportion of $CD8^+$ T cells found in the lymph nodes retained a naive unactivated phenotype (data not shown). The activation profiles of $CD8^+$ T cells in the lungs were similar to that of $CD4^+$ T cells (data not shown), indicating that both T cell subsets were efficiently primed in the lymph nodes upon mycobacterial infection.

Recently, we reported that numbers of $CD44^{high}CD8^+$ T cells in the lungs increase substantially by the second week of infection (14). We examined concomitant change in two activation/memory markers, CD44 and CD45RB, on the surface of $CD8^+$ T cells in the lungs in a time course experiment. By the second week of infection, a majority of $CD8^+$ T cells in the lungs up-regulated CD44 molecule with concurrent down-regulation of CD45RB molecule, and this phenotype remained stable for at least 4 wk postinfection (Fig. 1*C*), indicating establishment of the effector/memory phenotype. $CD4^+$ T cells in the lungs undergo similar changes with >90% T cells acquiring the $CD44^{high}CD45RB^{low}$ phenotype (data not shown). Thus, the number of lung $CD8^+$ T cells displaying a true naive phenotype in the lungs of infected mice was negligible as early as 2 wk postinfection. In the lungs, a substantial increase (8–10-fold) in the percentage of $CD69^+$

CD8$^+$ T cells, as compared with uninfected control, was observed by 2 wk postinfection; by 4 wk postinfection, ~50% of CD8$^+$ T cells were CD69$^+$ (Fig. 1*D*). Starting from 2 wk postinfection, the percentage of CD8$^+$ T cells in the lungs expressing CD69 was equal to that of CD4$^+$ T cells (data not shown), supporting the hypothesis that both T cell subsets are functioning during *M. tuberculosis* infection. These data suggest that effector CD8$^+$ T cells are not only recruited to the lung during infection, but also actively recognize mycobacterial Ags and engage in the ongoing immune response at this site.

Overall, the data presented in this study indicate that lungs and lung-draining lymph nodes contain large numbers of activated effector CD8$^+$ T cells and are a likely source for mycobacteria-specific CTLs. To enrich for Ag-specific CD8$^+$ T cells, lung and lymph node cells were cultured with murine bone marrow-derived *M. tuberculosis*-infected MHC class II$^{-/-}$ DCs in the presence of IL-2. Because these DCs are unable to present Ags to CD4$^+$ T cells, by day 5 the resultant lung and lymph node T cell cultures were comprised of 70–85% CD8$^+$ T cells (data not shown). Therefore, 3–5-fold enrichment in CD8$^+$ T cells was observed in the in vitro cultures as compared with the infected lungs in which CD8$^+$ T cells did not exceed 20–25% of total lymphocyte population (14). We were not able to grow CD8$^+$ T cells from the lungs of uninfected mice, which indicates that culture conditions were not optimal for priming of naive CD8$^+$ T cells and that only CD8$^+$ T cells previously primed for mycobacterial Ags were expanded during the culture period (data not shown).

Cytotoxic activity of CD8$^+$ T cells cultured from lungs and lung-draining lymph nodes of infected mice

Cytotoxic activity of CD8$^+$ lung and lymph node T cells harvested from mice 4 wk postinfection was tested after 5 days of coculture with *M. tuberculosis*-infected MHC class II$^{-/-}$ DCs. Bone marrow-derived Mϕ uninfected or infected with live *M. tuberculosis* for 18 h before CTL assay were used as targets in a 4-h ^{51}Cr release assay. Both lung and lymph node CD8$^+$ T cells lysed infected Mϕ, albeit with low efficiency (Fig. 2*A*). Similar results were obtained with lung and lymph node cells harvested from mice 2, 3, 5, and 6 wk postinfection (data not shown). We routinely observed low nonspecific lysis of uninfected Mϕ by lung CTLs and somewhat higher nonspecific lysis by lymph node CTLs. This might be due to the priming of naive lymph node cells to foreign Ags, such as FBS Ags, during the short stimulation with DCs. Since the majority of lung T cells had an activated/effector phenotype and presumably defined Ag specificity before ex vivo stimulation, priming of naive cells during short-term culture was negligible.

The low efficiency of specific lysis by the CD8$^+$ T cells may have been due to inefficient Ag processing by the infected Mϕ. We sought to improve the target cells by allowing more time for Ag processing and presentation. Cytotoxic activity of lung CD8$^+$ T cells was tested using as targets Mϕ infected for 18–20 or 42–44 h. In several independent experiments, the longer interaction between Mϕ and *M. tuberculosis* bacilli led to a substantial increase in the specific lysis by lung CD8$^+$ T cells (Fig 2*B* and data not shown). There was no significant increase in MHC class I expression on Mϕ at 42 h as compared with 18 h postinfection (data not shown). The improvement in lysis efficiency by CD8$^+$ CTLs was most likely due to an increase in the pool of available Ags or more efficient presentation of those Ags with the longer infection period.

The possibility existed that *M. tuberculosis*-infected Mϕ differ from uninfected Mϕ in their susceptibility to lysis by CTLs. Because this infection is cytotoxic to some degree, infected Mϕ might be lysed more readily, which would resemble low level specific lysis. To exclude this possibility, dose-dependent lysis of uninfected and infected Mϕ by purified perforin was assessed in a 2-h ^{51}Cr release assay. No significant difference was observed between purified perforin-mediated lysis of uninfected and infected Mϕ (Fig. 2*C*), indicating that infection does not lead to a more easily lysed Mϕ.

CD8$^+$ CTLs are present in the lungs and lymph nodes of mice infected via aerosol route

In the experiments described above, mice were infected with *M. tuberculosis* i.v. Because infection with *M. tuberculosis* naturally occurs via the respiratory route, we tested whether the CTLs were also present in the lungs and lung-draining lymph nodes of mice infected via aerosol. When mice were infected via aerosol, a delay in the development of CD8$^+$ T cell-mediated immune response in the lungs was observed, as compared with the immune response following i.v. infection. However, by 6 wk postinfection, the activation parameters of CD8$^+$ T cells in the lungs of aerosol-infected mice were similar to those of CD8$^+$ T cells from the lungs of i.v. infected mice (data not shown). Lung and lymph node cells harvested from aerosol-infected mice were stimulated for 5 days, as described above, and tested for cytotoxic activity against Mϕ infected with *M. tuberculosis* for 42 h (Fig. 3). The lysis of infected Mϕ targets by these CD8$^+$ CTLs was similar to the lysis by CTLs from i.v. infected mice, indicating that priming of CD8$^+$ CTLs specific for mycobacterial Ags and their subsequent migration to and function in the lungs occur regardless of infection route.

M. tuberculosis-*specific CD8$^+$ CTLs from lungs of infected mice are MHC class I restricted and lyse in a perforin-dependent manner*

In the murine model, the protective CD8$^+$ T cell-mediated immune response is believed to result from the recognition of Ags presented in the context of MHC class I molecules (5, 6). To examine the nature of Ag presentation in our system, β_2m-deficient (β_2m$^{-/-}$) Mϕ were used as targets. Specific lysis of *M. tuberculosis*-infected β_2m$^{-/-}$ Mϕ by lung and lymph node CTLs was reduced 75–100%, compared with wild-type Mϕ, in several independent experiments (Fig. 4, *A* and *B*). The small degree of lysis observed with infected β_2m$^{-/-}$ targets was most likely due to residual levels of MHC class I molecules detected in β_2m$^{-/-}$ mice. Thus, lysis was dependent on β_2m. However, β_2m is a component of both MHC class I and CD1 Ag-presenting complexes. In our experiments, Mϕ routinely expressed high levels of MHC class I molecules and only marginal levels of CD1d molecules (data not shown). Genes for type 1 CD1 molecules (CD1a-c) are not present in the mouse system. In three independent experiments, anti-MHC class I Ab cocktail inhibited specific lysis by lung CD8$^+$ CTLs by 63%, 53%, and 59% (Fig. 4*C*). MHC-mismatched Mϕ were also used as target cells. Bone marrow Mϕ from BALB/c mice (H-2d) were used as targets for CD8$^+$ T cells derived from C57BL/6 (H-2b) mice. Lysis of H-2d targets was reduced 66% compared with H-2b targets (Fig. 4*D*). Conversely, addition of anti-CD1d Ab had no effect on the lysis by lung CTLs (Fig. 4*E*). This does not exclude CD1d as a possible restricting element for CD8 T cell-mediated recognition of mycobacterial Ags, because the very weak CD1d expression observed on infected Mϕ might not have been enough to trigger TCR stimulation. However, the majority of cells recovered from the lungs in these studies appear to be MHC class I restricted.

To determine the contribution of granule-dependent lysis in the CTL assays, CD8$^+$ T cell cultures were incubated with strontium ions (Sr^{2+}) for the final 12 h of culture. This treatment induces degranulation of cytotoxic lymphocytes (33, 34), and thus inhibits

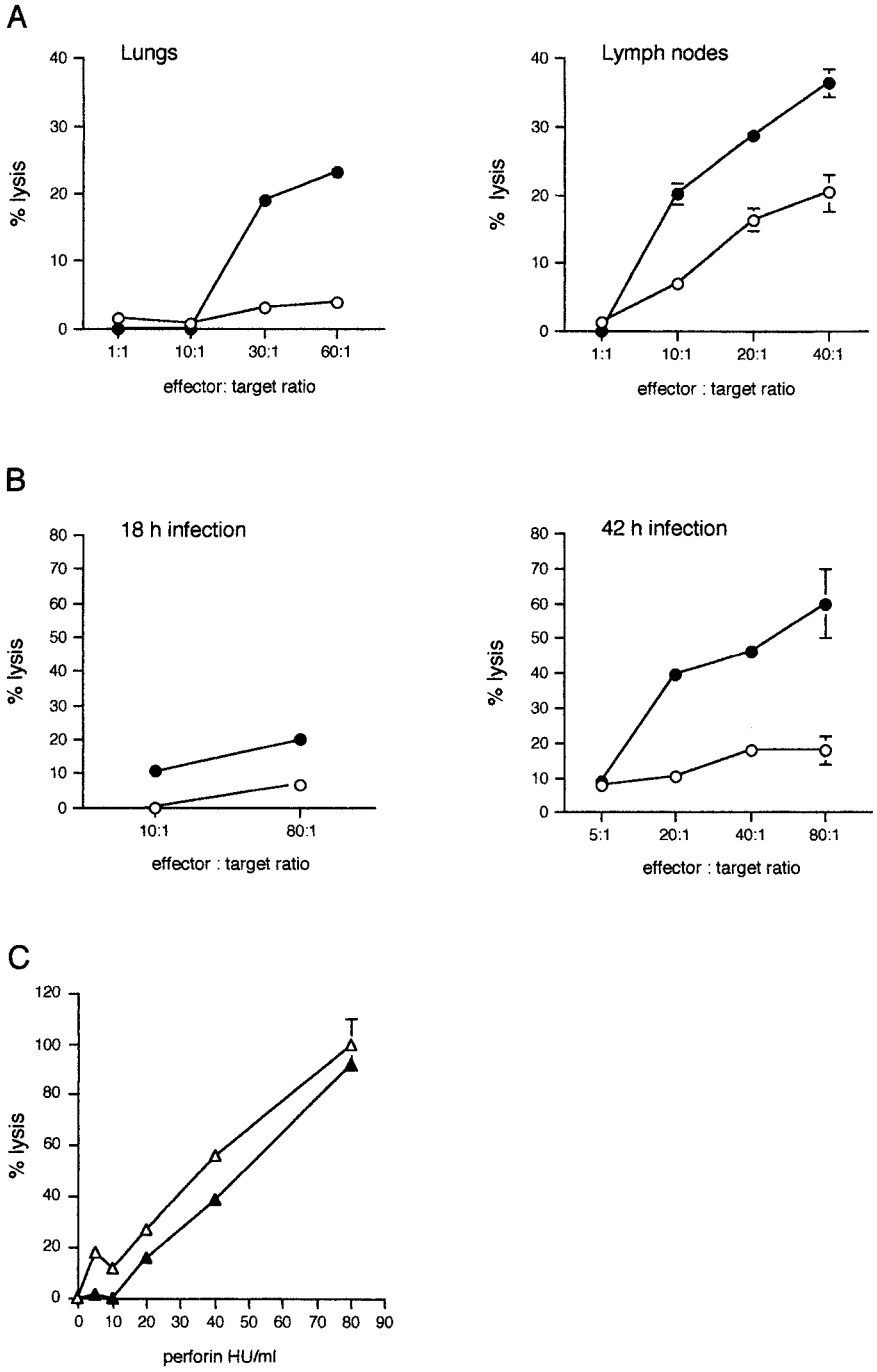

FIGURE 2. Cytotoxic activity of lung and lymph node $CD8^+$ T cell cultures. *A*, Lung and lymph node cells from three mice infected for 4 wk were pooled, stimulated with *M. tuberculosis*-infected MHC class $II^{-/-}$ DCs for 5 days, and used in a 4-h ^{51}Cr release cytotoxicity assay. Targets: uninfected Mϕ (○) and Mϕ infected for 18 h (●). Shown is a representative experiment of six experiments performed using cells from mice infected for 2, 3, 4, or 5 wk. *B*, Lung cells from 2-wk infected mice were cultured for 6 days, as described above, and used in a 4-h ^{51}Cr release assay against Mϕ either uninfected (○) or infected for 18 or 42 h (●). The experiment was repeated three times. *C*, Mϕ either uninfected (△) or infected with *M. tuberculosis* for 44 h (▲) were incubated with increasing concentrations of purified perforin, and lysis was assessed after 2 h. Error bars represent SE.

perforin-mediated lysis. The cytotoxic activity of $CD8^+$ T cell cultures was significantly (>80%) inhibited after incubation with Sr^{2+} (Fig. 4*F*), indicating that lysis of *M. tuberculosis*-infected Mϕ by lung CTL occurs via a granule-dependent pathway.

Perforin expression in the organs of infected mice

Because lysis of infected Mϕ by cultured $CD8^+$ CTLs was perforin dependent, we sought to confirm that perforin was expressed in the organs during infection. Immunohistochemistry was performed to assess perforin expression in the tissues of mice infected for 3 wk with *M. tuberculosis*. Lung and liver sections of uninfected mice contained very few lymphocytes, and perforin expression was not detected in these organs (data not shown). Spleen sections from uninfected mice did not stain positively for perforin either, even though this organ contains large numbers of lymphocytes (data not shown). In contrast, perforin expression was readily detected in the organs of infected mice. In the lungs, perforin-expressing cells were distributed throughout the lymphocytic infiltrates (Fig. 5, *A* and *B*). In the liver sections, perforin-expressing lymphocytes were clearly observed within the granulomas, in the lymphocytic rim surrounding the Mϕ core (Fig. 5, *C* and *D*). Perforin staining was also evident in the spleen sections of infected mice, albeit at lower frequency (data not shown). That fewer lymphocytes expressed perforin in the spleen correlates well with our observations that at least 45% of splenic $CD8^+$ T cells maintain an unactivated phenotype during acute infection (unpublished data). The majority of perforin expression was in small lymphocytic cells and was concentrated unipolarly in the cells, suggesting that they

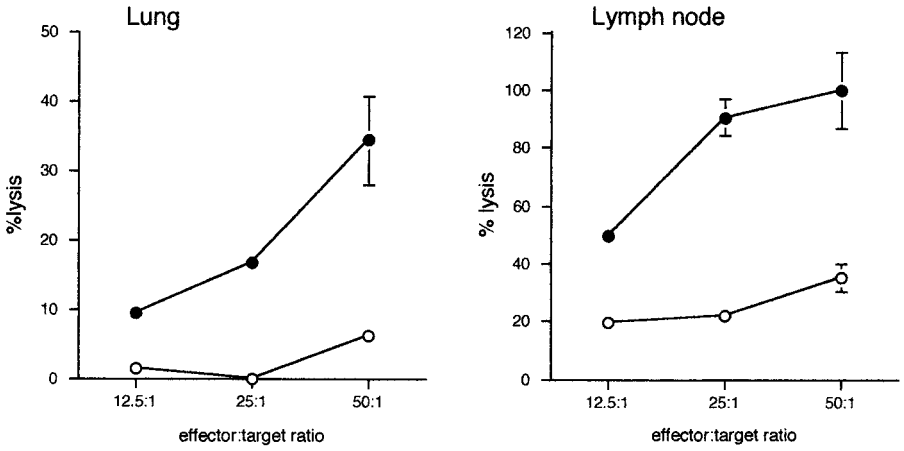

FIGURE 3. Cytotoxic activity of lung and lymph node CTLs generated after aerosol infection. C57BL/6 mice were infected via aerosol with ~100 viable *M. tuberculosis* bacilli. Lung and lymph node cells were harvested 6 wk postinfection and cultured as in Fig. 2. Cytotoxic activity of cultured cells was tested in a 4-h ^{51}Cr release assay using Mϕ either uninfected (○) or infected with *M. tuberculosis* for 42 h (●) as targets. The experiment was repeated twice. Error bars represent SE.

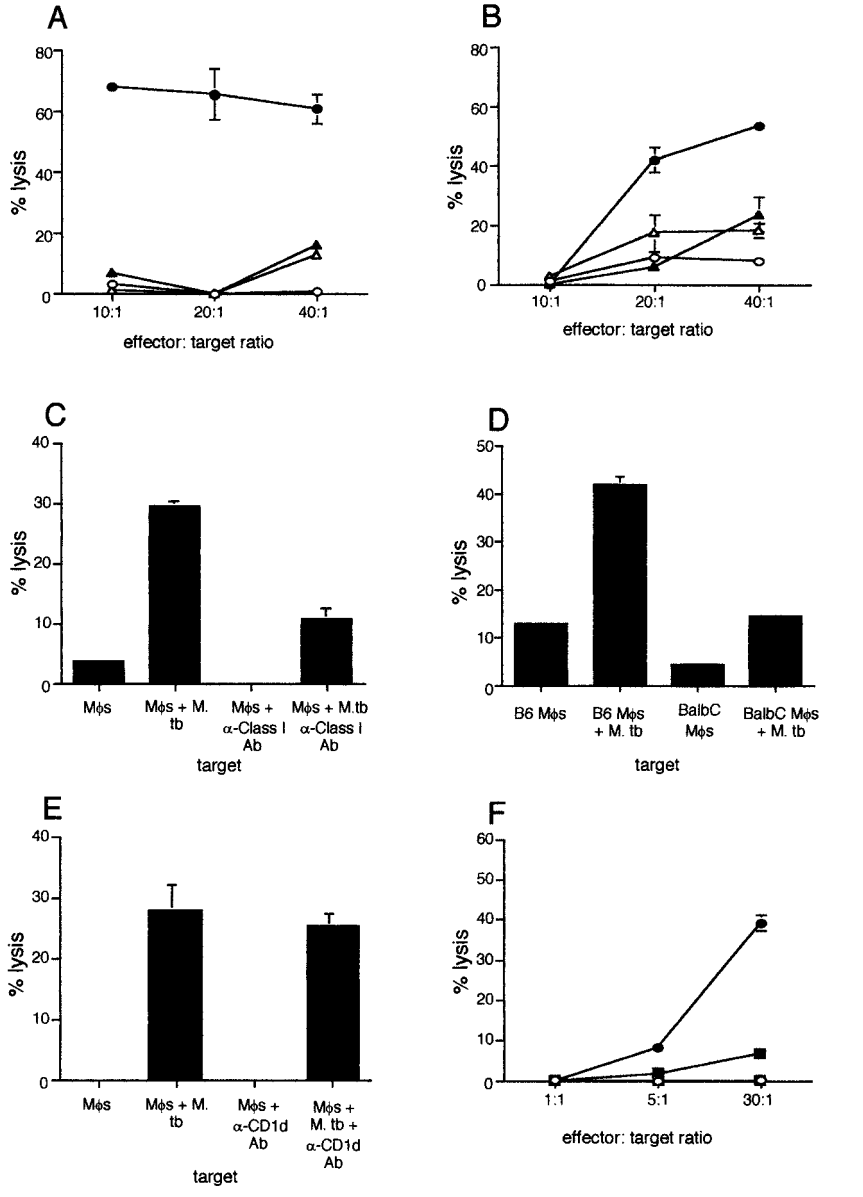

FIGURE 4. Mechanism of lysis by CD8$^+$ CTLs from lungs and lymph nodes of *M. tuberculosis*-infected mice. *A* and *B*, Lungs and lymph nodes were harvested from three to four mice infected i.v. for 2–4 wk, pooled, and cultured with infected DCs for 5–7 days. In all experiments shown (*A–F*), Mϕ infected for 36 h (filled symbols) and uninfected (open symbols) were used as targets. Ability of lung (*A*) and lymph node (*B*) CTLs to lyse wild-type Mϕ (circles) or β_2m$^{-/-}$ Mϕ (triangles) was examined in a 4-h ^{51}Cr release assay. The experiment was repeated five times. *C*, Cytotoxic activity of lung T cell cultures was tested in the absence or presence of anti-MHC class I Ab cocktail. Abs were added at the beginning of the CTL assay. Lysis at 40:1 E:T ratio is shown. The experiment was repeated three times. *D*, C57BL/6 (H-2d) or BALB/c (H-2b) Mϕ were used as targets. Lysis at 25:1 E:T ratio is shown. The experiment was repeated twice. *E*, Cytotoxic activity of lung T cell cultures was tested in the absence or presence of anti-CD1d Ab. Experiment was repeated three times. *F*, Lungs from mice infected for 6 wk via aerosol were harvested, pooled, and cultured, as described above. To test dependence of lysis on perforin, Sr^{2+} was added to T cell cultures at 15 mM (squares), or T cells were left untreated (circles). The experiment was repeated three times. Error bars represent SE.

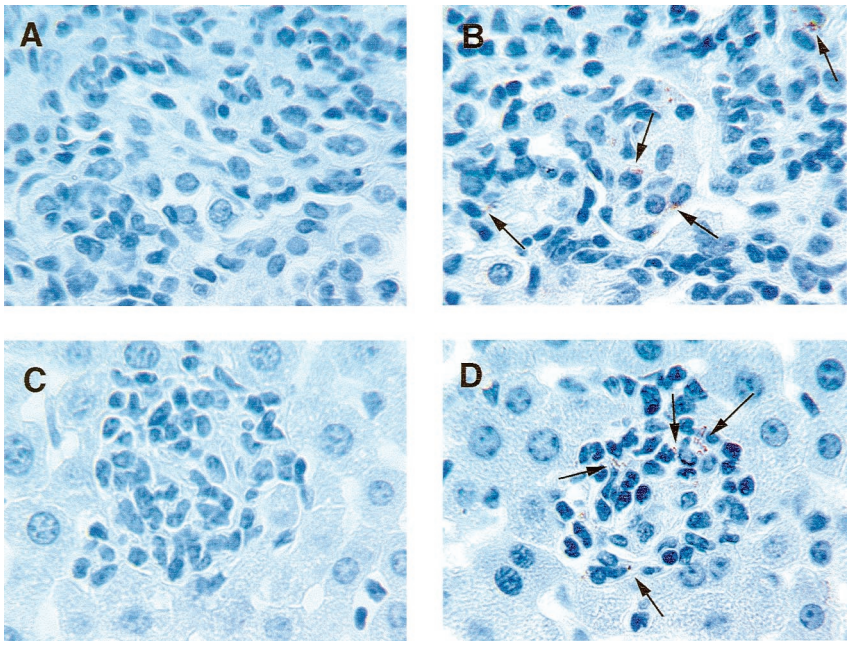

FIGURE 5. Perforin expression in the organs of infected mice. Organs of mice infected i.v. with *M. tuberculosis* were harvested 3 wk postinfection. Lung (*A* and *B*) and liver (*C* and *D*) sections were stained with anti-perforin (*B* and *D*) or isotype control (*A* and *C*) Abs. Black arrows show perforin staining.

were activated lymphocytes (Fig. 5, *B* and *D*). A number of cells expressed only few granules in the cytoplasm, but cells were also detected that expressed large perforin aggregates.

We reported previously that granulomas in the lungs of infected mice contained both CD4 and $CD8^+$ T cells (5). However, the Abs specific for these molecules are only useful for staining fresh frozen tissue sections. In the present studies, we used paraformaldehyde-fixed tissue for immunohistochemistry, to reduce the biohazard risks of preparing tissue sections. Thus, it was not possible to perform double staining on tissue sections to confirm that the $perforin^+$ cells were also $CD8^+$. Thus, to identify the $perforin^+$ cells, we performed intracellular staining and FACS analysis. $CD8^+$ T cells freshly harvested from the lungs of mice infected for 3, 4, or 5 wk were positive for perforin (Fig. 6*A*). In contrast, no perforin staining was observed in the $CD4^+$ T cell subset (Fig. 6*B*).

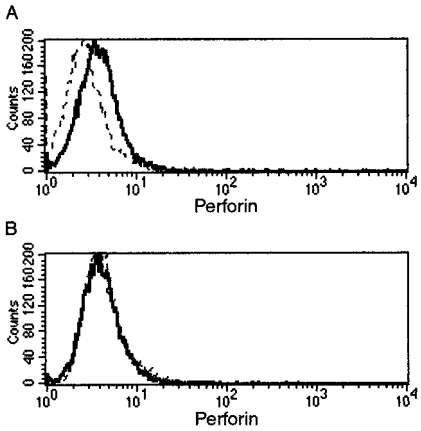

FIGURE 6. Differential perforin expression by $CD4^+$ and $CD8^+$ T cells from lungs of infected mice. Lung cells were harvested 3 wk postinfection, fixed in paraformaldehyde, permeabilized with 0.3% saponin, and stained with anti-perforin Ab (solid line) or isotype control (broken line), followed by staining with anti-CD8 and anti-CD4 Abs. Cells were gated on CD8 (*A*) or CD4 (*B*), and perforin expression within each gate was analyzed. The experiment was repeated four times.

Perforin deficiency alters the activation state of $CD8^+$ T cells

Previously, it was demonstrated that single deficiencies in perforin, granzyme B, or FAS receptor molecules did not affect short-term survival of *M. tuberculosis*-infected mice (25, 26). The interpretation of these results was that the cytotoxic activity of $CD8^+$ T cells does not contribute to protective immunity during tuberculosis. Recently, perforin deficiency was shown to have a profound effect on the development of the $CD8^+$ T cell-mediated immune response during lymphocytic choriomeningitis virus infection; $CD8^+$ T cells were in a heightened state of activation in $P^{-/-}$ mice as compared with the wild-type mice (35). We examined whether perforin deficiency affected development of $CD8^+$ T cells during the course of *M. tuberculosis* infection. By 4 wk postinfection, $P^{-/-}$ mice had increased numbers of lymphocytes in the lungs, attributable to a doubling in the numbers of $CD8^+$ T cells (Fig. 7*A*). Percentages of $CD8^+$ $CD69^+$ and $CD8^+$ $CD25^+$ T cells were also increased in the lungs of infected $P^{-/-}$ mice compared with wild-type mice (Fig. 7*B*), suggesting that $CD8^+$ T cells at the site of infection were in a state of hyperactivation. Additionally, mean fluorescent intensity of CD25 expression on the surface of $P^{-/-}$ $CD8^+$ T cells was increased ~2-fold (data not shown), suggesting enhancement in the activation state of individual cells. No changes were observed in the numbers or activation state of $CD4^+$ T cells in $P^{-/-}$ mice as compared with the wild-type mice (data not shown).

To compare the cytokine production potential of T cells from $P^{-/-}$ and wild-type mice during *M. tuberculosis* infection, we stimulated lung cells with anti-CD3 and anti-CD28 mAbs for 5 h in the presence of monensin and performed intracellular cytokine staining for IFN-γ and TNF-α. The total number of IFN-γ-secreting $CD8^+$ T cells in the lungs of $P^{-/-}$ mice was increased 4–4.5-fold as compared with the wild-type mice (Fig. 7*C*), in accordance with data published previously (26). A significant increase was also observed in the numbers of TNF-α-secreting $CD8^+$ T cells (data not shown). To assess cytokine production by T cells at the site of infection, we cultured fresh lung cells in the presence of monensin for 5 h without ex vivo Ab stimulation. As described previously (14), in the absence of stimulation, 5–8% of wild-type $CD8^+$ T cells secreted IFN-γ. In contrast, $P^{-/-}$ $CD8^+$ T cells

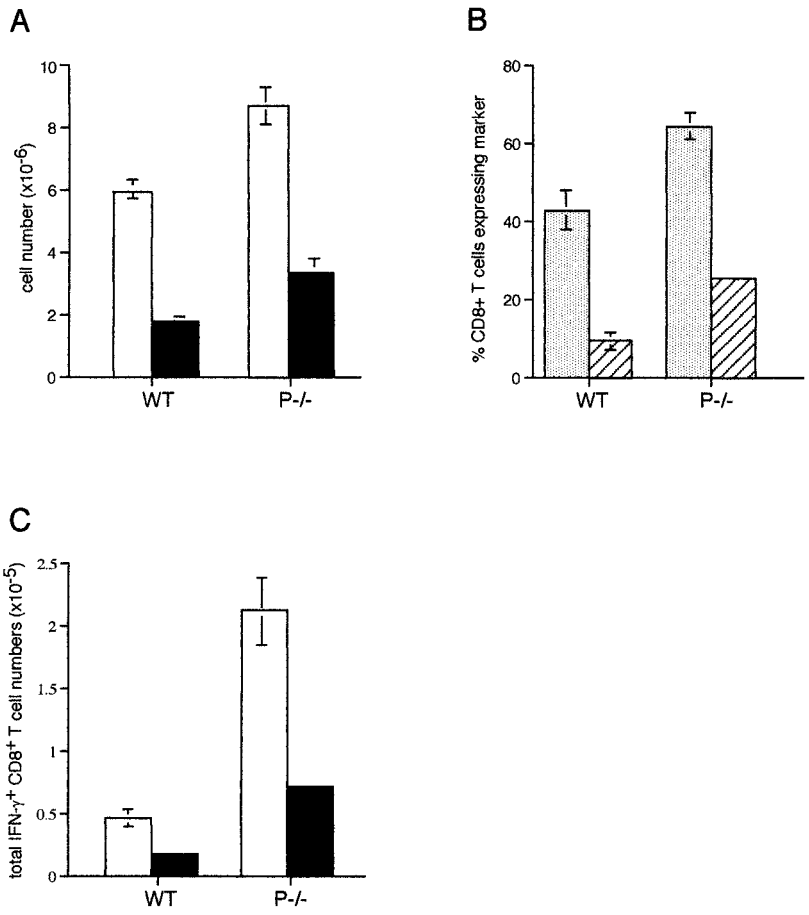

FIGURE 7. Changes in the $CD8^+$ T cell composition of and cytokine production by $P^{-/-}$ lungs. Lung cells from wild-type and $P^{-/-}$ mice infected with *M. tuberculosis* were harvested 4 wk postinfection. *A*, Total numbers of lymphocytic cells (□) were determined by trypan blue exclusion. Cells were stained for CD8 and analyzed by flow cytometry, and total numbers of $CD8^+$ T cells in the lymphocyte gate were determined (■). *B*, Cells were stained for CD8, CD69, and CD25; fixed in paraformaldehyde; and subjected to two-color flow cytometry. Cells were gated on CD8, and expression of CD69 (▨) and CD25 ([▨) within the gate was analyzed. Each bar represents 8–15 mice. Error bars represent SE. *C*, Cells were stimulated with anti-CD3 and anti-CD28 Abs (□) or left unstimulated (■) for 5–6 h in the presence of monensin. Cells were stained with CD8, fixed in paraformaldehyde, permeabilized, and stained for intracellular IFN-γ. Cells were gated on CD8 and analyzed by two-color flow cytometry. For $P^{-/-}$ cells, $p < 0.05$ as compared with wild-type controls. Each bar represents two to eight mice. Error bars represent SE.

readily secreted IFN-γ without stimulation, and the total number of IFN-γ^+ $CD8^+$ T cells in these cultures was increased ~4-fold (Fig. 7*C*). Cytokine production by $CD4^+$ T cells from $P^{-/-}$ mice with or without Ab stimulation was unchanged as compared with the wild-type control (data not shown).

Discussion

It is increasingly obvious that control of tuberculosis does not depend exclusively on the $CD4^+$ T cell-mediated immune response. A growing body of evidence suggests that $CD8^+$ T cells actively participate in the protective immune response to *M. tuberculosis*. However, the precise role of $CD8^+$ T cells during tuberculosis remains unclear. We hypothesized that $CD8^+$ T cells function as cytotoxic lymphocytes to lyse *M. tuberculosis*-infected Mϕ in tissues. In this study, we present evidence that *M. tuberculosis*-specific $CD8^+$ CTL are present in the lungs and lung-draining lymph nodes of mice infected by either the aerosol or i.v. route with virulent *M. tuberculosis*. These cells express perforin in vivo and lyse *M. tuberculosis*-infected Mϕ in an MHC class I-dependent manner.

Studies of the cytotoxic function of mycobacteria-reactive $CD8^+$ T cells have been hampered by difficulties in demonstrating the presence of $CD8^+$ CTLs in the organs of *M. tuberculosis*-infected mice. Various groups have reported the generation of $CD8^+$ T cells by immunization strategies involving killed or attenuated mycobacteria, or mycobacterial Ags (17, 19, 20, 36–39). These cells were demonstrated to recognize mycobacterial Ags in a MHC class I or MHC-independent manner. Following infection with live *M. tuberculosis*, $CD8^+$ T cells specific for epitopes of the 38-kDa glycolipoprotein were isolated (20, 36). However, with the exception of hsp65-specific CTLs generated by immunization with a cell line expressing this Ag (19), no other reports document the ability of $CD8^+$ CTLs to lyse Mϕ infected with live *M. tuberculosis*, which is an important component of protection by CTLs. Ags recognized by $CD8^+$ T cells could be generated during infection in a variety of ways, including by infected Mϕ or DCs, or extracellular bacterial proteins or apoptotic fragments of infected Mϕ taken up and processed by DCs. It is important to demonstrate that the $CD8^+$ T cells specific for mycobacterial Ags can actually recognize infected Mϕ in a functional manner, because these are the cells that must be attacked by the $CD8^+$ T cells to provide protection. This study is the first to report the isolation of $CD8^+$ CTL from the lungs of mice infected with virulent *M. tuberculosis* that specifically lyse Mϕ infected with live *M. tuberculosis*.

Several possible explanations exist as to why *M. tuberculosis*-specific $CD8^+$ CTLs have been so difficult to detect in infected mice. One hypothesis was that $CD8^+$ T cells were not primed during infection. A number of recent studies providing evidence that activated $CD8^+$ T cells that produce cytokine in response to mycobacterial Ags appear in the lungs following infection argue against this hypothesis (13, 14). Most studies on CD8 CTL responses in tuberculosis mouse models focused on the immune responses in the spleen. We focused on $CD8^+$ T cells present in the lungs and lung-draining lymph nodes of infected mice. In the lungs of infected mice, ≥90% of $CD8^+$ T cells acquired an effector/memory phenotype by 14 days postinfection. By 4 wk postinfection, ~50% of $CD8^+$ T cells in the lungs were expressing CD69, suggesting active participation of this cell subset in the ongoing immune response. Similar changes were also observed in the lung-draining lymph nodes, with a significant enrichment in the $CD8^+$

T cells and CD69$^+$ CD8$^+$ T cells, indicating that CD8$^+$ T cells were undergoing priming to mycobacterial Ags. The phenotype of the CD8$^+$ T cells in infected lungs and draining lymph nodes suggested that these organs were highly enriched for effector CD8$^+$ T cells and were most likely to contain *M. tuberculosis*-specific CTL.

The choice of infected DCs for brief in vitro restimulation of the CD8$^+$ T cells also contributed to the success in detecting mycobacteria-specific CD8 CTL. The number of mycobacterial Ags recognized by CD8$^+$ T cells is unknown. We reasoned that restimulation with DCs infected with live *M. tuberculosis* rather than cells transfected with the gene for one potential Ag would allow expansion of a more complete pool of specific CD8$^+$ T cells. In other studies, bone marrow or peritoneal derived Mϕ infected with live or killed *M. tuberculosis* or transfected with mycobacterial genes were used as stimulators in CD8$^+$ T cells cultures. However, infected Mϕ might not be well suited for this role because they can be suppressive (40, 41). In our hands, bone marrow-derived *M. tuberculosis*-infected Mϕ were poor at stimulating mycobacteria-specific proliferation of splenocytes[4] or IFN-γ production from lung CD4 or CD8$^+$ T cells (14) from infected mice. In human studies, DCs were successfully used to expand or prime mycobacteria-specific CD8$^+$ T cells (11, 22). Infection of murine DCs with bacteria enhanced biosynthesis, stabilization, and surface expression of MHC class I molecules, suggesting effective presentation of intracellular bacterial Ags to CD8$^+$ T cells (42). We reported that *M. tuberculosis* infection of murine and human DCs resulted in maturation of the cells and enhanced stimulation of mycobacteria-specific T cell responses (14, 43).[4] Using DCs deficient in MHC class II molecules, we obtained lung and lymph node T cell cultures highly enriched for CD8$^+$ T cells ($\geq$70%) after 5–7 days of stimulation. The brevity of the culture period was intended to minimize priming of naive CD8$^+$ T cells that might be present in the lungs and lymph nodes and to reduce the potential effects of IL-2.

In human studies, low level cytotoxic activity of CD8$^+$ T cells from bronchoalveolar lavage of purified protein derivative-positive individuals against *M. tuberculosis*-infected monocytes was reported (21). In our murine studies, Mϕ infected with *M. tuberculosis* for 42–44 h were much better targets for CTL than those infected 12–18 h, suggesting that processing and presentation of mycobacterial Ags by Mϕ are not efficient. At present, it is not clear how mycobacterial Ags are processed and presented by MHC class I molecules during infection. Presentation of soluble OVA in the context of MHC class I molecules was facilitated by virulent *M. tuberculosis* (44), and it has been suggested that Mϕ are capable of transferring Ags from phagosomal compartments into the cytosol (45). Presentation of this type might require a longer time for generation of Ags presented to CD8$^+$ T cells than is required during infection with a cytosolic pathogen. Previously, we demonstrated that Mϕ infected for 24 or 48 h did not stimulate the secretion of significant levels of IFN-γ by CD8$^+$ and CD4$^+$ T cells (14). The fact that specific CD8-mediated lysis of Mϕ, albeit low, was observed after a short (18-h) infection period suggests that, in vivo, cytotoxicity might be the earliest elicited T cell response.

Although lysis of *M. tuberculosis*-infected Mϕ by cultured CD8$^+$ T cells demonstrated that mycobacteria-specific CD8$^+$ CTLs are present in the lungs and lymph nodes of infected mice, it does not prove that cytotoxic activity is exerted by CD8$^+$ T cells in vivo. However, perforin molecules were readily detected in cells in lungs, livers, and spleens of infected mice and not in the organs of uninfected mice; perforin$^+$ cells were also CD8$^+$. It is not surprising that perforin expression was detected in relatively few lymphocytes in lung sections. CD8$^+$ T cells in the lungs comprise only 20–25% of the total lymphocyte population (14), and it is possible that not all activated CD8$^+$ T cells express perforin. Also, some CD8$^+$ T cells might be depleted of perforin after recent encounter with infected Mϕ. Once CD8$^+$ CTL recognize the antigenic determinant presented by infected Mϕ, the release of perforin- and granzyme-containing granules is triggered, resulting in target lysis. Having demonstrated that CD8$^+$ T cells from lungs of infected mice are capable of recognizing Ags presented by *M. tuberculosis*-infected Mϕ and lysing the infected Mϕ in a perforin-dependent manner, the finding that these cells express perforin in vivo strongly implicates participation of CD8$^+$ CTLs in the immune response.

In human studies, alternative MHC class I presentation to CD8$^+$ T cells (12) and presentation through CD1 or other nonpolymorphic MHC class Ib (11, 22, 24, 46) have been reported. In the murine model, the protective CD8$^+$ T cell-mediated immune response is β_2m and TAP dependent (5, 6). The data presented in this study indicate that CD8$^+$ CTLs from lungs recognized mycobacterial Ags presented by MHC class I molecules, and this recognition resulted in lysis of infected Mϕ. Addition of anti-CD1d Ab did not affect lysis by CTLs. However, we cannot rule out the possibility that CD1-restricted cells were present in our cultures because expression of CD1d molecule by infected Mϕ was difficult to detect. In any case, type I CD1 molecules are not present in mice, so the contribution of CD1a-c presentation cannot be tested in this system.

The involvement of cytotoxic CD8$^+$ T cells in the protective immune response to *M. tuberculosis* has been challenged by findings that mice with single deficiencies in perforin-, granzyme B-, and FAS receptor-deficient mice were shown to be resistant to the infection (25, 26). However, it is becoming clear that other lytic pathways can successfully compensate a deficiency in any single cytotoxic mechanism. Clearance of influenza virus mediated either by perforin- or FAS-dependent mechanisms was reported (47). Additionally, partial protection of mice from *Listeria monocytogenes* by single Ag-specific P$^{-/-}$ CD8$^+$ T cells was demonstrated to require TNF-α production by these cells (48). Thus, the fact that P$^{-/-}$ mice do not succumb to infection does not necessarily rule out the involvement of cytotoxic perforin-mediated responses during the normal course of infection. Recently, perforin has been suggested to down-modulate the CD8$^+$ T cell responses during persistent viral infection (35). We observed a substantial increase in CD8$^+$ T cells in the lungs of P$^{-/-}$ mice following *M. tuberculosis* infection, and cytokine secretion by CD8$^+$ T cells in the lungs was also enhanced (~4-fold, compared with wild-type mice). These results suggested that perforin plays a role in regulating CD8 T cell proliferation and function during *M. tuberculosis* infection as well. Failure of CD8$^+$ T cells to quickly lyse infected targets might result in the prolonged interaction between these cells and infected Mϕ, inducing cytokine production by CD8$^+$ T cells. In addition, these data suggest that lack of perforin-mediated killing by CD8$^+$ T cells may be compensated for by enhanced cytokine production by this cell subset. The enhanced cytokine responses of CD8$^+$ T cells might be protective during the acute stage of disease, but they also might induce pathology at the chronic stage of disease. Recent data indicate that P$^{-/-}$ mice do not survive as long as wild-type mice (49). Our data also suggest that perforin-mediated cytotoxicity is exerted during the normal course of *M. tuberculosis* infection and is at least partially responsible for reduced cytokine responses of CD8$^+$ T cells. This down-modulating effect of perforin is specific for the CD8$^+$ T cell subset.

In summary, we have demonstrated for the first time that the lungs of mice infected with *M. tuberculosis* via either the i.v.

or aerosol route contain CD8$^+$ CTLs with specificity for mycobacterial Ags. These CD8$^+$ T cells lysed Mϕ infected with live *M. tuberculosis* in an MHC class I- and perforin-dependent fashion and expressed perforin in vivo. Our data provide evidence that cytotoxic CD8$^+$ T cells responses are operative during the course of *M. tuberculosis* infection. In light of these and other recent data, the importance of CD8$^+$ T cells in the protective response against this pathogen cannot be neglected with respect to vaccine design. For a vaccine to be effective against tuberculosis, it appears that specific CD8$^+$ T cells, in addition to other cell subsets, must be induced. Whether IFN-γ production and cytotoxic activity of CD8$^+$ T cells are both important functions of CD8$^+$ T cells during infection remains to be determined.

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References

1. Orme, I., and F. Collins. 1984. Adoptive protection of the *Mycobacterium tuberculosis*-infected lung. *Cell. Immunol. 84:113.*
2. Orme, I. 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis. J. Immunol. 138:293.*
3. Muller, I., S. Cobbold, H. Waldmann, and S. H. E. Kaufmann. 1987. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of L3T4$^+$ and Lyt2$^+$ T cells. *Infect. Immun. 55:2037.*
4. Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-γ, yet succumb to tuberculosis. *J. Immunol. 162:5407.*
5. Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA 89:12013.*
6. Behar, S. M., C. C. Dascher, M. J. Grusby, C. R. Wang, and M. B. Brenner. 1999. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis. J. Exp. Med. 189:1973.*
7. Lampe, M. F., C. B. Wilson, M. J. Bevan, and M. N. Starnbach. 1998. γ Interferon production by cytotoxic T lymphocytes is required for resolution of *Chlamydia trachomatis* infection. *Infect. Immun. 66:5457.*
8. Harty, J. T., R. D. Schreiber, and M. J. Bevan. 1992. CD8 T cell can protect against an intracellular bacterium in an interferon γ-independent fashion. *Proc. Natl. Acad. Sci. USA 89:11612.*
9. Orme, I., E. Miller, A. Roberts, S. Furney, J. Griffen, K. Dobos, D. Chi, B. Rivoire, and P. Brennan. 1992. T lymphocytes mediating protection and cellular cytolysis during the course of *Mycobacterium tuberculosis* infection. *J. Immunol. 148:189.*
10. Lalvani, A., R. Brookes, R. Wilkinson, A. Malin, A. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and A. Hill. 1998. Human cytolytic and interferon γ-secreting CD8$^+$ T lymphocytes specific for *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA 95:270.*
11. Lewinsohn, D., M. Alderson, A. Briden, S. Riddell, S. Reed, and K. Grabstein. 1998. Characterization of human CD8$^+$ T cells reactive with *Mycobacterium tuberculosis*-infected antigen presenting cells. *J. Exp. Med. 187:1633.*
12. Canaday, D. H., C. Ziebold, E. H. Noss, K. A. Chervenak, C. V. Harding, and W. H. Boom. 1999. Activation of human CD8$^+$ $\alpha\beta$ TCR$^+$ cells by *Mycobacterium tuberculosis* via an alternate class I MHC antigen-processing pathway. *J. Immunol. 162:372.*
13. Feng, C. G., A. G. D. Bean, H. Hool, H. Briscoe, and W. J. Britton. 1999. Increase in γ interferon-secreting CD8$^+$, as well as CD4$^+$, T cells in lungs following aerosol infection with *Mycobacterium tuberculosis. Infect. Immun. 67: 3242.*
14. Serbina, N. V., and J. L. Flynn. 1999. Early emergence of CD8$^+$ T cells primed for production of type 1 cytokines in the lungs of *Mycobacterium tuberculosis*-infected mice. *Infect. Immun. 67:3980.*
15. Tascon, R. E., E. Stavropoulos, K. V. Lukacs, and M. J. Colston. 1998. Protection against *Mycobacterium tuberculosis* infection by CD8$^+$ T cells requires production of γ interferon. *Infect. Immun. 66:830.*
16. Kaufmann, S. H., U. Vath, J. E. Thole, J. D. Van Embden, and F. Emmrich. 1987. Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur. J. Immunol. 17:351.*
17. De Libero, G., I. Flesch, and S. H. E. Kaufmann. 1988. Mycobacteria-reactive Lyt-2$^+$ T cell lines. *Eur. J. Immunol. 18:59.*
18. Koga, T., A. Wand-Wurttenberger, J. DeBruyn, M. E. Munk, B. Schoel, and S. H. Kaufmann. 1989. T cells against a bacterial heat shock protein recognize stressed macrophage. *Science 245:1112.*
19. Silva, C. L., M. F. Silva, R. C. L. R. Pietro, and D. B. Lowrie. 1994. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat shock protein 65. *Immunology 83:341.*
20. Zhu, X., H. J. Stauss, J. Ivanyi, and H. M. Vordermeier. 1997. Specificity of CD8$^+$ T cells from subunit-vaccinated and infected H-2b mice recognizing the 38 kDa antigen of *Mycobacterium tuberculosis. Int. Immunol. 9:1669.*
21. Tan, J. S., D. H. Canady, W. H. Boom, K. N. Balaji, S. K. Schwander, and E. A. Rich. 1997. Human alveolar T lymphocyte responses to *Mycobacterium tuberculosis* infection. *J. Immunol. 159:290.*
22. Stenger, S., R. Mazzaccaro, K. Uyemura, S. Cho, P. Barnes, J. Rosat, A. Sette, M. Brenner, S. Porcelli, B. Bloom, and R. Modlin. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science 276:1684.*
23. Mohagheghpour, N., D. Gammon, L. M. Kawamura, A. van Vollenhoven, C. J. Benike, and E. G. Engleman. 1998. CTL response to *Mycobacterium tuberculosis*: identification of an immunogenic epitope in the 19-kDa lipoprotein. *J. Immunol. 161:2400.*
24. Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-uszynski, A. Melian, C. Bogdan, S. A. Porcelli, B. R. Bloom, A. M. Krensky, and R. L. Modlin. 1998. An antimicrobial activity of cytotoxic T cells mediated by granulysin. *Science 282:121.*
25. Cooper, A. M., C. D'Souza, A. A. Frank, and I. M. Orme. 1997. The course of *Mycobacterium tuberculosis* infection in the lungs of mice lacking expression of either perforin- or granzyme-mediated cytolytic mechanisms. *Infect. Immun. 65: 1317.*
26. Laochumroonvorapong, P., J. Wang, C.-C. Liu, W. Ye, A. L. Moreira, K. B. Elkon, V. H. Freedman, and G. Kaplan. 1997. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. *Infect. Immun. 65:127.*
27. Slifka, M. K., F. Rodriguez, and J. L. Whitton. 1999. Rapid on/off cycling of cytokine production by virus-specific CD8$^+$ T cells. *Nature 401:76.*
28. Persechini, P. M., and J. D.-E. Young. 1988. The primary structure of the lymphocyte pore-forming protein perforin: partial amino acid sequencing and determination of isoelectric point. *Biochem. Biophys. Res. Commun. 156:740.*
29. Zheng, L.-M., D. M. Ojcius, C.-C. Liu, M. D. Kramer, M. M. Simon, E. L. Parr, and J. D.-E. Young. 1991. Immunogold labelling of perforin and serine esterases in granulated metrial gland cells. *FASEB J. 5:79.*
30. Arva, E., and B. Andersson. 1999. Kinetics of cytokine release and expression of lymphocyte cell-surface activation markers after in vitro stimulation of human peripheral blood mononuclear cells with *Streptococcus pneumoniae. Scand. J. Immunol. 49:237.*
31. Biselli, R., P. M. Matricardi, R. D'Amelio, and A. Fattorossi. 1992. Multiparametric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. *Scand. J. Immunol. 35:439.*
32. Mardiney, M. R., M. R. Brown, and T. A. Fleisher. 1996. Measurement of T-cell CD69 expression: a rapid and effectively means to assess mitogen- or antigen-induced proliferative capacity in normals. *Cytometry 26:305.*
33. Neighbour, P. A., H. S. Huberman, and Y. Kress. 1982. Human large granular lymphocytes and natural killing ultrastructural studies of strontium-induced degranulation. *Eur. J. Immunol. 12:588.*
34. Quan, P. C., T. Ishizaka, and B. R. Bloom. 1982. Studies on the mechanism of NK cell lysis. *J. Immunol. 128:1786.*
35. Matloubian, M., M. Suresh, A. Glass, M. Galvan, K. Chow, J. K. Whitmire, G. M. Walsh, W. R. Clark, and R. Ahmed. 1999. A role for perforin in downregulating T-cell responses during chronic viral infection. *J. Virol. 73:2527.*
36. Zhu, X., N. Venkataprasad, H. S. Thangaraj, M. Hill, M. Singh, J. Ivanyi, and M. H. Vordermeier. 1997. Function and specificity of T cells following nucleic acid vaccination of mice against *Mycobacterium tuberculosis* infection. *J. Immunol. 158:5921.*
37. Bonato, V. L. D., V. M. F. Lima, R. E. Tascon, D. B. Lowrie, and C. L. Silva. 1998. Identification and characterization of protective T cells in hsp65 DNA-vaccinated and *Mycobacterium tuberculosis*-infected mice. *Infect. Immun. 66: 169.*
38. Denis, O., A. Tanghe, K. Palfliet, F. Jurion, T.-P. van der Berg, A. Vanonckelen, J. Ooms, E. Saman, J. B. Ulmer, J. Content, and K. Huygen. 1998. Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4$^+$ and CD8$^+$ T-cell epitopic repertoire broader than that stimulated by *Mycobacterium tuberculosis* H37Rv infection. *Infect. Immun. 66:1527.*
39. Da Fonseca, D. P. A. J., D. Joosten, R. van der Zee, D. L. Jue, M. Singh, H. M. Vordermeier, H. Snippe, and A. F. M. Verheul. 1998. Identification of new cytotoxic T-cell epitopes on the 38-kilodalton lipoglycoprotein of *Mycobacterium tuberculosis* by using lipopeptides. *Infect. Immun. 66:3190.*
40. Hirsch, C. S., R. Hussain, Z. Toosi, G. Dawood, F. Shahid, and J. J. Ellner. 1996. Cross-modulation by transforming growth factor β in human tuberculosis: suppression of antigen-driven blastogenesis and interferon γ production. *Proc. Natl. Acad. Sci. USA 93:3193.*

41. Hirsch, C. S., J. J. Ellner, R. Blinkhorn, and Z. Toossi. 1997. In vitro restoration of T cell responses in tuberculosis and augmentation of monocyte effector function against *Mycobacterium tuberculosis* by natural inhibitors of transforming growth factor β. *Proc. Natl. Acad. Sci. USA 94:3926.*
42. Rescigno, M., C. Citterio, C. Thery, M. Rittig, D. Medaglini, G. Pozzi, S. Amigorena, and P. Ricciardi-Castagnoli. 1998. Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc. Natl. Acad. Sci. USA 95:5229.*
43. Henderson, R. A., S. C. Watkins, and J. L. Flynn. 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J. Immunol. 159:635.*
44. Mazzaccaro, R. J., M. Gedde, E. R. Jensen, H. M. van Santem, H. L. Ploegh, K. L. Rock, and B. R. Bloom. 1996. Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA 93:11786.*
45. Kovacsovics-Bankowski, M., and K. L. Rock. 1995. A phagosome to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science 267:243.*
46. Beckman, E. M., S. A. Porcellis, C. T. Morita, S. M. Bahar. S. T. Furlong, and M. B. Brenner. 1994. Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. *Nature 372:691.*
47. Topham, D. J., R. A. Tripp, and P. C. Doherty. 1997. $CD8^+$ T cells clear influenza virus by perforin or FAS-dependent processes. *J. Immunol. 159:5197.*
48. White, D. W., and J. T. Harty. 1998. Perforin-deficient $CD8^+$ T cells provide immunity to *Listeria monocytogenes* by a mechanism that is independent of CD85 and IFN-γ but requires TNF-α. *J. Immunol. 160:898.*
49. Sousa, A. O., R. J. Mazzaccaro, R. G. Russell, F. K. Lee, O. C. Turner, S. Hong, L. Van Kaer, and B. Bloom. 1999. Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. *Proc. Natl. Acad. Sci. USA. 97:4204.*