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Mycobacterium tuberculosis-Specific CD8+ T Cells Require Perforin to Kill Target Cells and Provide Protection In Vivo1

Joshua S. Woodworth, Ying Wu, and Samuel M. Behar2

Optimal immunity to Mycobacterium tuberculosis (Mtb) infection requires CD8+ T cells, and several current Mtb vaccine candidates are being engineered to elicit enhanced CD8+ T cell responses. However, the function of these T cells and the mechanism by which they provide protection is still unknown. We have previously shown that CD8+ T cells specific for the mycobacterial Ags CFP10 and TB10.4 accumulate in the lungs of mice following Mtb infection and have cytolytic activity in vivo. In this study, we determine which cytolytic pathways are used by these CD8+ T cells during Mtb infection. We find that Mtb-specific CD8+ T cells lacking perforin have reduced cytolytic capacity in vivo. In the absence of perforin, the residual cytolytic activity is CD95 and TNFR dependent. This is particularly true in Mtb-infected lung tissue where disruption of both perforin and CD95 eliminates target cell lysis. Moreover, adoptive transfer of immune CD8+ T cells isolated from wild-type, but not perforin-deficient mice, protect recipient mice from Mtb infection. We conclude that CD8+ T cells require perforin-mediated cytolysis to protect animals from infection. These data show that CD8+ T cell-mediated protection during Mtb infection requires more than the secretion of IFN-γ and specifically defines the CD8+ cytolytic mechanisms utilized and required in vivo. The Journal of Immunology, 2008, 181: 8595–8603.

An adaptive cellular immune response is required for the control of Mycobacterium tuberculosis (Mtb) infection and for the efficacy of current vaccine strategies against Mtb. In addition to a requirement for CD4+ T cells in immunity to Mtb, the culmination of numerous studies has established that class I MHC-restricted CD8+ T cells are also required for optimum immunity following Mtb infection (reviewed in Ref. 1). Recognition of the protective value of CD8+ T cells has influenced the development of several Mtb vaccines, many of which are specifically designed to elicit Mtb-specific CD8+ T cells in hopes of increasing their protective efficacy (2–4). Indeed, there is now direct evidence that vaccine-elicited CD8+ T cells reduce bacterial loads in vaccinated mice providing proof of principle to these strategies (5, 6).

Although the requirement for Mtb-specific CD8+ T cells is appreciated, the mechanism by which these cells provide protection has not been defined. Several studies find that CD8+ T cells play distinct and nonredundant roles in immunity to Mtb. For example, depletion of CD8+ T cells, but not CD4+ T cells, impairs bacterial control in the Cornell model of latent Mtb infection (7). The biological basis for unique roles played by CD8+ T cells is likely to arise from several defining characteristics of these T cells. CD8+ T cells can recognize cells that lack class II MHC or suboptimally present class II MHC-restricted Ags and consequently cannot be detected by CD4+ T cells. This scenario can occur since Mtb inhibits class II MHC Ag presentation by macrophages, as well as infects and replicates within nonprofessional APCs such as human type II lung epithelial cells (8–10). The protective effect of CD8+ T cells may also require specific effector functions. Following Ag stimulation, Mtb-specific CD8+ T cells from infected animals and people produce cytokine mediators known to be required for optimal resistance to Mtb, including IFN-γ and TNF-α (11–16). In addition to their release of soluble mediators, CD8+ T cells can also act as CTL.

CD8+ T cells cultured from Mtb-infected humans and animals are cytolytic in vitro and CD8+ T cells localized within pulmonary Mtb lesions in humans and isolated from infected mice express perforin (Pfn) (13, 17–20). Recently, the use of well-defined peptide epitopes and class I MHC tetramers has lead to a greater understanding of Mtb-specific CD8+ T cells. Peptide stimulation of CD8+ T cells specific for the Mtb Ag TB10.4 leads to surface expression of the degranulation markers CD107A and CD107B and increased expression of CD95L (FasL), indicating their CTL potential (14). Moreover, pulmonary TB10.4-specific CD8+ T cells analyzed directly ex vivo inversely express surface CD107A and CD107B and intracellular granzyme B, suggesting that they are actively degranulating in vivo (11). Finally, we and others have shown that CD8+ T cells specific for several Mtb epitopes kill peptide-loaded target cells in vivo (12, 14, 21). Thus, upon TCR stimulation, CD8+ T cells are capable of cytolytic activity via release of cytotoxic granules and possibly other mechanisms such as cross-linking of CD95 (Fas) on target cells.

Based on the findings described above, one would predict that the molecular pathways that mediate the cytotoxic activity of CD8+ CTL would be critical for host resistance to Mtb infection.
However, studies comparing the bacterial burdens and survival of mice genetically deficient in different components of the known cytolytic pathways have produced conflicting results (22–25). One interpretation is that these pathways have a more critical role late during infection. For example, neither Ptprca Pep3b nor CD95-deficient (lpr) mice had a defect in immunity to Mtb during the early phase of infection (22). Similarly, Ptprca and granzyme B−/− mice do not show greater bacterial burdens following aerosol infection (22, 23). However, mice deficient in CD95/CD95L-mediated killing have increased bacterial burden during the chronic phase of pulmonary Mtb infection (24). Lastly, Mtb-infected Pfn−/− mice have impaired control of bacterial replication late during infection as well as reduced survival following infection (22, 25). Although there is not a clear consensus, it appears that the requirement for these molecular pathways in protective immunity may be dependent upon the route of infection and stage of disease.

The lack of a clear consensus from these studies is not surprising since compensatory changes in genetically deficient mice can confound evaluation of these individual cytolytic pathways. Specifically, the pathways themselves may be redundant and importantly CD95/CD95L and Pfn both affect immunoregulation of the T cell immunity. For example, lpr and gld mice have dysregulated lymphoproliferation and enhanced compensatory cytokine responses (22). Similarly, Pfn regulates the expansion and activation CD8+ T cells in many infectious models, including Mtb infection where Pfn−/− mice have an altered cytokine profile and increased numbers of CD8+ IFN-γ-producing T cells in their lungs (13, 22, 26). Thus, cytotoxic function and immunomodulation mediated by CD95/CD95L and Pfn can have opposing effects on host resistance, and evaluation of mice with genetic defects in these pathways may have unpredictable changes in susceptibility to Mtb infection. Finally, determining whether perforin and CD95L-deficient mice are more susceptible to Mtb infection cannot address whether these molecules are specifically required for the effect function of CD8+ T cells since perforin and CD95L are expressed by other cell types in addition to CD8+ T cells (27, 28).

We have previously shown that Mtb-specific CD8+ T cells elicited during infection have cytolytic activity in vivo. In the present study, we test the hypothesis that CD8+ T cell-mediated protection against Mtb infection requires this cytolytic activity. To do this, we use well-defined class I MHC-restricted peptide epitopes and in vivo CTL assays to define the mechanism(s) by which Mtb-specific CD8+ T cells kill target cells during infection. By contrasting CD8+ T cell killing with heretofore unreported in vivo CTL activity of class II MHC-restricted Mtb-specific CD4+ T cells, we show that the cytotoxic pathways identified are specific for CD8+ T cell function. Finally, we test the molecular pathways identified by the in vivo CTL assay using a T cell transfer model to determine whether the cytotoxic activity of CD8+ T cells is required for protection against Mtb infection.

**Materials and Methods**

**Mice**

Age-matched female C57BL/6J (B6) and syngeneic mice deficient in Pfn (C57BL6-Prf1tm1Sdz/J; Pfn−/−), TNFRp55 (C57BL6-Tnfrsf1a tm1Imx/J; TNFR−/−), CD95 (B6.MRL-Fas−/−; lpr), CD95L (B6Smn.C3-Tnf5f601d/J; gld) or congenic for CD45.1 (B6.SJL-Ptprca Pep3b/J) and B10.BR-H2d Tg-Tb10.4 (B6.SJL-Ptprca Pep3b/J; B10.BR;Ptprca Pep3b/J; lpr) were purchased from The Jackson Laboratory. Pfn−/− mice were backcrossed two generations to B10.BR mice to generate Pfn-deficient H-2d animals (B10.BR;Pfn−/−). Mice were housed in a biosafety level 3 facility under specific pathogen-free conditions at the Animal Biocontainment Hazard Suite (Dana-Farber Cancer Institute, Boston, MA) and were used in a protocol approved by the institution.

**Bacteria and aerosol infections**

All infections were performed using virulent Mtb (Erman strain). For each infection, a bacterial aliquot was thawed, sonicated twice for 10 s in a cup horn sonicator, and then diluted in 0.9% NaCl-0.02% Tween 80. A 15-ml suspension of Mtb was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies). Mice were infected via the aerosol route using a nose-only exposure unit (Intox Products) and received 100–200 CFU/mouse (11, 12, 29).

**Isolation of cells**

Single-cell suspensions were prepared by forcing spleens through a 70-μm nylon strainer (Fishier). The RBC were lysed using lysis buffer (0.15 M NaCl, 1 mM KHCO3, and 0.1 mM sodium EDTA, pH 7.3). After washing, the cells were resuspended in complete medium (RPMI 1640, 10% FCS, 2% HEPES, 1% l-glutamine, 1% penicillin-streptomycin, and 0.1% 2-ME). Lung mononuclear cells were obtained by digesting tissue with collagenase type IV (Sigma-Aldrich) for 2 h at 37°C followed by filtration through a 60-mesh metal strainer and 70-μm nylon strainer. Lung lysis was performed as described above. Cells were enumerated in 4% trypan blue with a hemacytometer.

**Peptides**

Mtb peptides TB10.4.1–11 (MYNNYPAM), ESAT6.15 (MTEQW NFAGIEAAA) (30), Mtb32.90–102 (GAPINSATAM) (16), CFP10.39–48 (VESTAGSL) (12), and CFP10.25–32 (LAEQAGNFERISGDL) (12) were commercially synthesized (BioSource International). The identity of peptide was confirmed by mass spectrometry. The peptides were dissolved in DMSO and stored at −20°C until used. Peptides for cytolytic and restimulation assays were used unpurified. The purity of peptides for tetramer production was >95%.

**In vivo cytotoxicity assay**

In vivo cytotoxicity was determined using peptide-coated splenocytes differentially labeled with the intracellular fluorescent dyes CFSE and cholomethyl-benzoyl-amino-tetramethylrhodamine (CMTMR; Molecular Probes) as previously described (12, 31). Splenocytes from uninfected mice were prepared to be used as targets as described above. When indicated, splenic B cells were isolated for use as targets using CD19 microbeads and LS columns (Miltenyi Biotech) as per the manufacturer’s protocol. Target cells were labeled with either 10 μM CMTMR in complete medium for 15 min at 37°C or CFSE (0.5 and 5.0 μM) for low and high or 0.3125, 1.25, and 5 μM for low, mid, and high) in PBS for 8 min at room temperature, followed by extensive washing. Target cell populations were pulsed with 10 μM of the appropriate peptide at 37°C for 1 h in complete medium left unpulsed. Labeled target populations were gated on to ratio and injected i.v. into age-matched infected and infected recipient mice (2 mice × 105 or 5 × 105 of each labeled population per mouse). For experiments involving backcrossed mice, uninfected B10.BR and B10.BR;Pfn−/− littermate controls were used. After 20 h, recipient spleens and lungs were harvested and single-cell suspensions were made as described above. Ratios of recovered CFSE- and CMTMR-labeled target lymphocyte populations were determined by flow cytometry. Percent specific killing was determined by the following formula: percent specific killing = 100 − [100 × (ratio in infected mice/ratio in uninfected mice)], where ratio = percent peptide-pulsed target cells/percent unpulsed target cells.

For comparison between multiple genetically deficient targets and recipients, killing was normalized to the specific killing of wild-type (WT) target cells in WT recipient mice. Therefore, we define percent maximal killing = (percent specific killing)/(average percent specific killing of WT targets in WT Mtb-infected recipients).

**Flow cytometry**

Spleen and lung cells were stained as previously described (11, 12) using Abs specific for mouse CD3ε, CD4, and CD8α conjugated to FITC, PE, PerCP, or allophycocyanin (BD Pharmingen). Tetrads were produced using CFP10.39–48 loaded H-2Kb, TB10.4.1–11 loaded H-2Kd, and Mtb32.90–102 loaded H-2Dd complexed to streptavidin(SA)-PE or SA-allophycocyanin (National Institutes of Health Tetramer Core Facility, Emory University Vaccine Center, Atlanta, GA). Cells were analyzed using a FACSCanto (BD Biosciences) and FlowJo analysis software (Tree Star). Lymphocytes were gated by size and granularity. Tetramer-positive staining was determined by gating on CD8-positive cells (and CFSE or CMTMR-negative cells when applicable) and tetramer-positive gates were established based on staining observed for uninfected mice and using SA-fluorochromes as a negative control. To reduce error from...
autofluorescence, recovered target cells from in vivo cytolytic assays were gated as CMTMR+ or CFSE+ lymphocytes.

Ab depletion

Depletion of CD4+ cells for in vivo cytolytic assays was achieved by serial i.p. injections of 0.5 mg of anti-CD4 Ab (clone GK1.5) 1 and 3 days before target cell injection.

CD8+ T cell adoptive transfer

Immune CD8+ T cells from spleens of Mtb-infected mice harvested 4 wk after aerosol infection were purified by two rounds of magnetic bead selection. Splenocytes were first subjected to CD8+ T cell isolation kit (negative selection) followed by CD8+ bead-positive selection using an autoMACS Pro Separator (Miltenyi Biotec). Cell purity was consistently ≥95% CD8+, with <0.05% CD4+ cells. Cells (5 × 10⁶) were injected i.v. into recipient mice that had been sublethally irradiated 24 h before with 600 rad from a cesium-137 source. Within 24 h of cell transfer, recipient mice were infected with aerosolized Mtb.

In vitro restimulation assays

The ELISPOT method was used to detect IFN-γ secretion by individual T cells from the lungs of infected mice following stimulation with peptides in vitro using the BD Biosciences ELISPOT Kit as previously described (12). Briefly, pulmonary T cells were purified from pooled tissue by CD90 microbeads using an autoMACS Pro Separator, cultured in triplicate with 100 U/ml rIL-2, and irradiated naive syngeneic splenocytes as APC. Cells were stimulated with 108 peptide for 20–24 h at 37°C. Postincubation, cells were discarded and plates were developed. The spots were enumerated using a series A Immunospot plate reader, Image Acquisition version 4.0 and Immunospot version 3.2 analysis software (Cellular Technology). Ag-specific spots were determined by subtracting the average media control values.

CFU determination

After euthanasia by CO2 inhalation, the left lungs and spleens were aseptically removed and individually homogenized in 0.9% NaCl-0.02% Tween 80 with a MiniBead Beater 8 (BioSpec Products). Viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates (Remel). Colonies were counted after 3 wk of incubation at 37°C.

Statistics

The Prism software program was used to perform all statistical analyses (GraphPad). CFU and tetramer staining data were log10 transformed before analysis. Statistical significance was analyzed using one-way ANOVA and Bonferroni’s multiple comparison posttest. When multiple target cell populations were used in the in vivo CTL assay, statistical testing was performed using a paired one-way ANOVA.

Results

TB10.44-specific CD8+ T cells are cytolytic in vivo

Our initial description of CFP10- and TB10.4-specific CD8+ CTL activity in vivo was in H-2b and H-2d MHC haplotype mice, respectively (12). Recently, Billeskov et al. (14) identified an epitope shared by the Mtb-secreted Ags TB10.3 and TB10.4 that is recognized by CD8+ T cells. The epitope, which is contained within amino acid residues 3–11, is H-2Kb restricted and TB10.44-specific CD8+ T cells have cytotoxic activity in vivo (14). The 8-mer TB10.44–11 has a higher predicted binding affinity to H-2Kb (a score of 98 vs 85 by RANKPEP; http://bio.dfci.harvard.edu/Tools/rankpep.html (32)), suggesting that TB10.44–11 is the minimal epitope recognized by TB10.4-specific CD8+ T cells in H-2b mice. Analysis of aerosol Mtb-infected B6 mice using TB10.44–11-loaded H-2Kb tetramers reveals a discrete population of CD8+ T cells that typically accounts for 30–40%, and occasionally >50%, of total lung CD8+ T cells during chronic Mtb infection (Fig. 1A). Lymphocytes isolated from Mtb-infected mice secrete IFN-γ upon TB10.44–11 peptide stimulation, confirming the stimulatory capacity of the 8-mer (data not shown). Finally, in a CFSE-based in vivo cytotoxicity assay, TB10.44–11-loaded target cells are specifically killed when transferred into Mtb-infected mice, confirming that TB10.44–11-specific CD8+ T cells are cytolytic in vivo (Fig. 1B). These data show that, similar to CFP10.22–39 in H-2b mice and TB10.420–28 in H-2d mice, TB10.44–11 is an immunodominant antigenic target of Mtb-specific CD8+ CTL in H-2b mice.

Mtb-specific CD8+ T cells utilize a hierarchy of cytolytic mechanisms in vivo

We sought to systematically investigate the molecular pathways required for Mtb Ag-specific CD8+ T cell cytotoxic activity in vivo. For this, we adapted the fluorescence-based in vivo cytotoxicity assay to include genetically deficient target cells and recipient animals. Because cytotoxic activity of CD8+ T cells elicited following viral infection is often dependent upon Pfn, we initially assessed whether the cytolytic activity of TB10.44–11-specific CD8+ T cells requires Pfn. Given that Pfn has an immunoregulatory role, and in particular mediates clonal expansion of Ag specific CD8+ T cells (13, 26), we enumerated the number of TB10.44–11-specific CD8+ T cells in each infected recipient. TB10.44–11-loaded target cells were killed more efficiently after transfer into Mtb-infected WT B6 mice compared with Pfn−/− mice (Fig. 2A). This effect was more pronounced when the specific killing was normalized by the number of TB10.44–11-specific CD8+ T cells in the spleen of each infected recipient mouse. Thus, despite a greater number of TB10.44–11-specific CD8+ T cells, TB10.44–11-pulsed target cells were killed less efficiently in Mtb-infected Pfn−/− mice (Fig. 2A). H-2Kb-restricted CFP10.22–39-specific CD8+ T cells were similarly dependent upon Pfn to efficiently kill CFP10.22–39 target cells, an effect that was more clearly observed after compensating for the increased number of CFP10.22–39-specific CD8+ T cells in the spleens of the Mtb-infected
B10BR.Pfn−/− mice following Mtb infection (Fig. 2B). These data demonstrate that Pfn is required for optimal cytotoxic activity of Mtb-specific CD8+ T cells elicited following respiratory Mtb infection. However, the residual cytolytic potential of CD8+ T cells from Mtb-infected mice suggested that these CD8+ T cells use additional molecular mechanisms to lyse target cells.

To further define the molecular pathways used by Mtb-specific CD8+ T cells to kill target cells, we focused on three well-described cytolytic pathways: 1) Pfn-mediated killing, 2) CD95/CD95L-mediated cytolysis, and 3) TNFRp55-induced cell death. WT B6, CD95-deficient (lpr), and TNFR−/− splenocytes were differentially labeled with CFSE and loaded with TB10.4-specific peptide, while a control population of WT cells was labeled with the orange fluorescent dye CMTMR, but not loaded with peptide. These target cell populations were then combined and transferred into uninfected or Mtb-infected WT and Pfn−/− mice. After 20 h, splenocytes were harvested and the relative ratio of the TB10.4-loaded vs control targets cells was determined by flow cytometry and the specific killing of each target population was calculated (Fig. 2C).

As described above (see Figs. 1B and 2A), TB10.4-loaded WT target cells are readily eliminated in Mtb-infected WT recipients, with an average specific killing of 81% (normalized to 100% in Fig. 2D). Following transfer into Mtb-infected WT recipients, TB10.4-loaded TNFR−/− targets are killed equally as well as WT targets, whereas TB10.4-loaded lpr targets are killed slightly less efficiently, with a nominal 4.1% reduction in relative killing (p < 0.001; Fig. 2D). Thus, in mice with intact Pfn-mediated cytotoxicity, there is only a limited contribution by other cytolytic pathways.

Although WT, TNFR−/−, and lpr target cells are all killed similarly in WT recipients, this is not the case following transfer into Mtb-infected Pfn−/− recipients. TB10.4-loaded WT targets are killed 26.7 ± 2.6% less efficiently in Pfn−/− recipients compared with infected WT recipients (p < 0.001) (100% vs 73.3%; Fig. 2D). In the absence of Pfn, TB10.4-loaded TNFR−/− and lpr targets were killed less efficiently than TB10.4-loaded WT targets after transfer into Mtb-infected WT mice. In fact, in Pfn−/− recipients, TNFR−/− and lpr targets were killed only 65 and 32% as efficiently as their WT counterparts in WT recipients, respectively (Fig. 2D). Taken together, these data show a predominant use of Pfn-mediated cytotoxicity by Mtb-specific CD8+ T cells that can be only partially compensated by the TNF and CD95L pathways.

Pfn-independent CD8+ T cell killing in lung tissue is highly CD95 dependent

TB10.4-specific CD8+ T cells accumulate in the lungs of Mtb-infected mice. Thus, it was of considerable interest to determine...
whether the cytotoxic mechanism used by highly activated pulmonary T cells differs from those in the spleen. To properly control for intrinsic differences in target cell survival and accurately calculate specific killing, the in vivo cytotoxicity assay was modified to include both TB10.44–11-loaded and unladen target cells of each genotype (i.e., WT, TNFR−/−, lpr). We found that 93 ± 1.2% (normalized to 100% in Fig. 3) of peptide-loaded WT target cells are specifically killed in WT lung tissue. Similar to our analysis in the spleen of infected mice (see Fig. 2), neither the absence of TNFR nor CD95 signaling has a significant effect on specific target cell killing in WT animals, yet WT target cells are consistently killed nearly 25% less efficiency in the lungs of Pfnt−/− mice (Fig. 3, A and B). TNFR signaling is dispensable for the killing of target cells in the lungs of Pfnt−/− mice, since WT and TNFR−/− target cells were killed similarly under these conditions (Fig. 3A). In contrast, only 11 ± 6.5% of the target cells are killed in the absence of both Pfnt and CD95, representing an 88 ± 7% reduction in maximal killing that is unparalleled in the spleen (cf Figs. 2D and 3B). Therefore, the relative hierarchy of Pfnt, CD95 and TNFR described for killing of target cells in the spleen is maintained in lung tissue; however, the absolute requirement for CD95L by CD8+ T cells in the absence of Pfnt is greatly increased at the pulmonary site of infection.

**Mtb-specific CD4+ T cells use a cytolytic mechanism distinct from CD8+ T cells**

Although CTL is synonymous with CD8+ T cells, CD4+ T cells can also be cytolytic. We have previously identified a class II MHC-restricted peptide from the Mtb Ag CFP10 (CFP1011–25) that is specifically recognized by CD4+ T cells during Mtb infection of H-2k mice (12). Using a CFSE-based in vivo cytotoxic assay, we found that purified B cells loaded with CFP1011–25 are readily killed in infected B10.BR animals (Fig. 4A). Notably, the specific killing of peptide-pulsed B cells is enhanced compared with that of CFP1011–25-loaded total splenocytes, presumably because the B cell targets are uniformly class II MHC+ and thus capable of being recognized by CFP1011–25-specific CD4+ T cells (data not shown). Importantly, the specific killing is blocked by treatment of the infected recipients with anti-CD4 mAb (Fig. 4, A and B).

We found that B cells loaded with the H-2k class II MHC-restricted epitope ESAT61–15 are selectively killed in Mtb-infected B6 mice (Fig. 4C) (30). Similar to CD8+ T cell killing, ESAT61–15-loaded TNFR-deficient target cells are killed with similar efficiency as WT targets, while lpr targets are killed slightly less efficiently in WT mice (Fig. 4D). Specific killing of ESAT61–15-loaded WT, TNFR−/−, and lpr targets was unaffected in CD95L-deficient (gld) mice (Fig. 4D). However, in contrast to our results for TB10.44–11-specific CD8+ CTL in which specific killing was significantly reduced in Pfnt−/− mice, none of the ESAT61–15-loaded target cell populations was killed less efficiently by CD4+ CTL in the absence of Pfnt (Fig. 4D). Thus, CD4+ CTL elicited by Mtb infection do not have the same dependence on Pfnt and CD95 that we observe for CD8+ CTL.

**FIGURE 3.** Cytolytic mechanisms used by pulmonary TB10.44–11-specific CD8+ T cells in vivo. A, 1:1:1:1 mixture of unpulsed CMTMR-labeled WT, TB10.44–11-pulsed CFSElow WT, unpulsed CFSEmed TNFR−/−, and TB10.44–11-pulsed CFSEhigh TNFR−/− targets cells were injected into uninfected and Mtb-infected B6 and Pfnt−/− mice. The lungs were analyzed to determine the relative TB10.44–11-specific killing of TNFR targets vs WT targets. B, Same as in A, but with lpr targets instead of TNFR−/− targets. Bar, mean ± SEM of four to five mice per group. ***, p < 0.001 by one-way ANOVA vs WT targets within the same recipient group or as indicated by the bracket.

**FIGURE 4.** Mtb-specific CD4+ T cell cytolytic activity in vivo. A, 1:1 mixture of unpulsed CFSEhigh and CFP1011–25-pulsed CFSElow-labeled splenic B cells were injected into uninfected B10.BR mice (left) and Mtb-infected mice untreated (center) or pretreated with anti-CD4 Ab (right). The percent specific killing of CFP1011–25-pulsed cells is indicated in B; Bar, mean ± SEM of four to five mice; ***, p < 0.0001 by Student’s t test. C, In vivo cytolytic assay of ESAT61–15-pulsed B cells was used to determine the percent ESAT61–15 specific killing in B6 mice infected with Mtb 5 wk before. Bar, mean ± SEM of five mice. Data are representative of three similar experiments. D, Unpulsed CMTMR-labeled WT, ESAT61–15-pulsed CFSElow WT, CFSEmed TNFR−/−, and CFSEhigh lpr splenic B cells were mixed 1:1:1:1 and injected into mice. Target cells recovered from the spleens of uninfected and Mtb-infected B6, Pfnt−/−, and gld mice were analyzed for specific killing. The percent maximal killing was calculated as described in Materials and Methods. Bar, mean ± SEM of three to five mice per group. Means were not significantly different when tested using a one-way ANOVA.
Adoptively transferred CD8\(^+\) T cells require Pfn to protect mice from Mtb

We next sought to determine whether the cytotoxic activity of CD8\(^+\) T cells is required for their ability to mediate protection against Mtb. A T cell adoptive transfer system was established to evaluate the molecular requirements of Mtb-specific CD8\(^+\) effector T cells. Highly purified CD8\(^+\) T cells from Mtb-infected mice were transferred into sublethally irradiated syngeneic mice. Following transfer, recipient mice were infected with Mtb by the aerosol route. Since even a small number of contaminating CD4\(^+\) T cells could possibly expand following infection and confound the interpretation of the results, we validated our model by transferring CD45.1 and CD45.2 expression by pulmonary CD4\(^+\) and CD8\(^+\) T cells was analyzed. Data are representative of five individual mice.

Protection by adoptively transferred immune CD8\(^+\) T cells was dependent on Pfn. We first evaluated whether the absence of Pfn affected the engraftment or expansion of Ag-specific CD8\(^+\) T cells during the time frame of the adoptive transfer experiment. Sublethally irradiated B6 mice received either no cells or highly purified WT or Pfn\(^{-/-}\) immune CD8\(^+\) T cells isolated from infected mice 4 wk after Mtb infection. Purified WT and Pfn\(^{-/-}\) T cells were each 95% CD8\(^+\) and IFN-\(\gamma\)-releasing CD8\(^+\) T cells were enumerated by ELISPOT. Bar, mean ± SEM of triplicate wells. Splenic (F) and pulmonary (G) Mtb bacterial burden was assessed 3 wk after no transfer or transfer of WT or Pfn\(^{-/-}\) CD8\(^+\) T cells and challenge with aerosolized Mtb. Bar, mean ± SEM of five mice per group; ns, Not significant; *, \(p < 0.05\) and **, \(p < 0.01\) by one-way ANOVA vs no transfer. The total number of T cells recovered from the lung was similar in all three groups (Fig. 5C). Mice that did not receive transferred cells had approximately equal proportions of CD4\(^+\) and CD8\(^+\) T cells, as we typically observe in nonirradiated Mtb-infected animals (our unpublished observation). In contrast, mice that received WT or Pfn\(^{-/-}\) CD8\(^+\) T cells had a higher CD8\(^+\).CD4\(^+\) T cell ratio.

FIGURE 5. Protection by adoptively transferred immune CD8\(^+\) T cells requires Pfn. A, B6 (CD45.2) mice were irradiated and injected i.v. with highly purified immune CD8\(^+\) T cells isolated from congeneric CD45.1 mice and then challenged with Mtb by the aerosol route within 24 h. Three weeks later, the CD45.1 and CD45.2 expression by pulmonary CD4\(^+\) and CD8\(^+\) T cells was analyzed. Data are representative of five individual mice. B, Splenic CFU from mice in A that received splenic CD8\(^+\) T cells from uninfected (naive) or infected (immune) mice or no cells (no transfer). Bar, mean. C and D, Three weeks after infection of mice that received no cells (No tx) or WT or Pfn\(^{-/-}\) CD8\(^+\) T cells, pulmonary cells were isolated, enumerated, and analyzed by flow cytometry. The total number of pulmonary CD4\(^+\) (□) and CD8\(^+\) (■) T cells (C) and the total number of TB10.4_(11)–specific (left panel) and Mtb3293–102–specific (right panel) CD8\(^+\) T cells (D) was determined by tetramer staining for each individual animal. Bar, mean ± SEM of five mice per group; *, \(p < 0.05\) by one-way ANOVA vs no transfer (E). Purified CD90\(^+\) T cells pooled from mice that received WT or Pfn\(^{-/-}\) CD8\(^+\) T cells (five mice per group) were stimulated with TB10.4_(11) and IFN-\(\gamma\) by ELISPOT. Bar, mean ± SEM of five mice per group. Uninfected T cells were each 95% CD8\(^+\) T cell population and verify the usefulness of this strategy to evaluate the protective function of Mtb-specific CD8\(^+\) effector T cells.

Since Pfn is required for CD8\(^+\) CTL function in vivo, we wished to investigate whether the protection against Mtb infection mediated by CD8\(^+\) T cells was dependent on Pfn. We first evaluated whether the absence of Pfn affected the engraftment or expansion of Ag-specific CD8\(^+\) T cells during the time frame of the adoptive transfer experiment. Sublethally irradiated B6 mice received either no cells or highly purified WT or Pfn\(^{-/-}\) immune CD8\(^+\) T cells isolated from infected mice 4 wk after Mtb infection. Purified WT and Pfn\(^{-/-}\) T cells were each 95% CD8\(^+\) with similar percentages of Mtb-specific cells based on tetramer analysis (data not shown).Recipient mice were infected by the aerosol route within 24 h after irradiation and adoptive transfer and analyzed 3 wk later. The total number of T cells recovered from the lung was similar in all three groups (Fig. 5C). Mice that did not receive transferred cells had approximately equal proportions of CD4\(^+\) and CD8\(^+\) T cells, as we typically observe in nonirradiated Mtb-infected animals (our unpublished observation). In contrast, mice that received WT or Pfn\(^{-/-}\) CD8\(^+\) T cells had a higher CD8\(^+\).CD4\(^+\) T cell ratio.
Mtb Ag-specific CD8+ T cells numbers were also significantly increased in mice that received CD8+ T cells, which further confirms the successful engraftment of transferred CD8+ T cells (Fig. 5D). Tetramer analysis revealed that mice that received WT or Pfn−/− CD8+ T cells had similar numbers of TB10.4 and Mtb32 Ag-specific CD8+ T cells 3 wk after infection (Fig. 5D). Mice that received WT or Pfn−/− CD8+ T cells had a similar frequency of TB10.4−/−specific CD8+ IFN-γ-producing T cells (Fig. 5E).

Therefore, differences in the protective ability of transferred WT and Pfn−/− CD8+ T cells is unlikely to be due to changes in the number of Mtb-specific T cells or their ability to produce IFN-γ. These data confirm that the transferred CD8+ cells survived and trafficked to the pulmonary site of infection in these animals.

To assess the protective capacity of WT and Pfn−/− CD8+ T cells, pulmonary and splenic bacterial loads were measured 3 wk after Mtb infection to quantify the protection afforded by the transferred CD8+ T cells. Compared with mice that did not receive T cells, recipients of WT CD8+ T cells had significantly reduced bacterial numbers with log10 reductions of 0.66 and 0.70 in the spleen and lung, respectively, indicating that the transferred WT CD8+ T cells provided protection (Fig. 5, F and G). In contrast, mice that received Pfn−/− CD8+ T cells had splenic and lung bacterial burdens statistically indistinguishable from those of mice that did not receive transferred cells and significantly higher than those of mice that received WT CD8+ T cells (Fig. 5, F and G). Thus, despite a similar number of Mtb-specific CD8+ T cells with similar potential to make IFN-γ, effector CD8+ T cells from Pfn−/− mice are unable to protect recipient mice against pulmonary Mtb infection. These data show that Pfn is absolutely required for the ability of CD8+ effector T cells to mediate protection against Mtb infection.

Discussion

Whether the cytolytic activity of CD8+ T cells is required for their protective function during Mtb infection has been difficult to determine. Studies using Pfn−/−, lpr and gld mice are problematic given the compensatory and immunoregulatory changes that occur in these mice. Studies using T cell clones and Mtb-infected cells in vitro have been informative, but are subject to artifacts from in vitro stimulation and cannot address the relevance of T cell-mediated cytolyis to host resistance to Mtb. Therefore, we used specific peptide epitopes and an in vivo CTLL assay to investigate the effector mechanisms acquired and required by Mtb CD8+ T cells elicited following aerosol Mtb infection.

We found that Pfn plays an important role in killing target cells in vivo. Importantly, simultaneous enumeration of Ag-specific CD8+ T cells revealed that the reduction in specific killing observed in Pfn−/− mice occurred despite a significant increase in the total number of Ag-specific CD8+ T cells. Although CD95- and TNFR-dependent killing was not detected in WT animals, a significant role for these pathways was revealed in the absence of Pfn. These data show that elimination of a single cytolytic pathway cannot completely abolish Mtb-specific CD8+ T cell cytolytic capacity in vivo. In fact, our data suggest the existence of a hierarchy of pathways that have both synergy and redundancy.

We have presented the first example of CD4+ T cell cytolytic activity detected in vivo following mycobacterial infection. Cytotoxicity mediated by allogeneic murine CD4+ T cells is CD95 dependent and lymphocytic choriomeningitis virus-specific CD4+ T cells elicited by infection have cytolytic activity in vivo that is partially dependent on CD95 and CD95L (33, 34). Similarly, hemagglutinin-specific CD4+ T cells primed in vitro kill target cells and protect mice against influenza by a Pfn-dependent mechanism (28). There is precedent for thinking that Mtb-specific CD4+ T cells are cytotoxic, because human Mtb-specific CD4+ T cells have Pfn-independent cytotoxicity against infected mononuclear cells (19, 35). We show that class II MHC-restricted Mtb-specific CD4+ T cells are cytotoxic in vivo and compare their molecular requirements with those of Mtb-specific CD8+ T cells. Although we were unable to elucidate a clear requirement for any one cytolytic pathway, perhaps because of mechanistic redundancy, our results show that CD4+ T cells have molecular requirements distinct from CD8+ T cells. Most notably, lack of Pfn does not impair Mtb-specific CD4+ T cell-mediated killing in vivo. This difference highlights the specificity of our assay system, as well as supports a functional relationship between CD8+ T cells and Pfn in Mtb infection.

Comparison of CD8+ T cell cytotoxicity in the spleen and lung tissue demonstrated quantitative differences in the molecular pathways used. In particular, there was a greater dependency on the CD95/CD95L pathway in the lung. This result may be relevant to the physiological functional capacity of pulmonary CTL during Mtb infection. For example, several factors including IFN-γ, can lead to up-regulation of CD95 in the tuberculoid lung and may lead to more efficient use of this pathway in killing both endogenous and adoptively transferred targets (36). Inflammatory cytokine mediators can also enhance class I MHC presentation and expression of cell adhesion molecules, which can enhance target cell recognition by CD8+ T cells. Alternatively, the mechanistic differences observed between the spleen and lung may be the consequence of the different E/T ratios. With a significantly greater number of Ag-specific CD8+ T cells and organized granulomas, conditions in the inflamed lung may favor utilization of cytolytic mechanisms that are less efficient in splenic tissue. Moreover, following aerosol infection, a larger proportion of splenic Mtb-specific CD8+ T cells have a central memory phenotype and these CD8+ T cells may differ in their use of cytolytic pathways (11).

In addition to defining the effector mechanisms acquired by CD8+ T cells in vivo, we wished to determine whether these pathways were required for protection. A limited number of studies have addressed the ability of adoptively transferred CD8+ T cells to protect mice from Mtb infection. Although T cell transfer into unmanipulated mice does not confer any additional protection, Orme and Collins (37) and Orme and Orme (38) demonstrated that adoptive transfer of either CD4- or CD8-enriched immune T cells protects mice that have been irradiated and thymectomized. Tascon et al. (49) observed that adoptive transfer of naive WT CD8+ T cells, but not IFN-γ-deficient CD8+ T cells, into nude mice provided protection against subsequent challenge with Mtb. These data have led many to conclude that CD8+ T cells require IFN-γ production to protect mice from Mtb infection. However, the protection observed by Tascon et al. (49) was in highly immunodeficient mice lacking many other potentially important and natural sources of IFN-γ including CD4+, NKT, and γδ T cells. Therefore, as the only source of IFN-γ, the need for IFN-γ production by CD8+ effector T cells may have been overestimated and the physiological mechanism by which CD8+ T cells mediate protection was masked. For example, because the adoptively transferred cells were naive CD8+ T cells, the requirement for IFN-γ may have reflected its role in T cell priming instead of effector function. Unfortunately, the relative efficiency with which naive WT and IFN-γ−/− CD8+ T cells generated Ag-specific T cells in Mtb-infected nude mice was not assessed and may have differed greatly. Additionally, IFN-γ can have large effects on relevant cells types in Mtb infection, including the induction of the immunoproteasome and up-regulation of class I MHC that may be required to sensitize targets to recognition by CD8+ CTL (39, 40). Thus, although the absolute requirement for IFN-γ in a productive
immune response to Mt is well known, it remains unclear how much IFN-γ is required or from which cell types it must be secreted in a protective response to Mt.

In our model, we adoptively transferred highly purified immune CD8+ T cells to study the protective mechanism of CD8+ T cells elicited following aerosol Mt infection that parallel the cells investigated in our cytolytic mechanistic studies. The source of donor CD8+ T cells used in these studies were the spleens of Mt-infected mice, which are composed of predominantly effector/effector memory and some central memory cells (11, 41). Although the frequency of splenic Ag-specific CD8+ T cells is much lower than the lung where most of the T cells have an effector phenotype, our ability to obtain highly purified populations of CD8+ T cells from the spleen is a critical aspect of this study. We used sublethally irradiated mice as recipients to promote the engraftment of the transferred cells, which we were able to directly confirm by flow cytometry and immunological assays. By the end of 3 wk, an endogenous CD4+ and CD8+ Mtb-specific T cell response to Mt infection was detected in all experimental groups, including irradiated mice that did not receive donor T cells (Fig. 5 and our unpublished observations). Therefore, our system allowed us to specifically address the protective effects of CD8+ cytolytic activity in subjects with an immune system containing a diverse repertoire of IFN-γ-secreting cells. The conclusions, therefore, are most likely applicable to CD8+ T cells during the acute phase of infection, and further study will be needed to see whether these data parallel the necessary effector requirements during chronic or latent infection.

A lack of Pfn completely abolished protection afforded by transferred immune CD8+ T cells, suggesting that Pfn-mediated cytosis is an essential and nonredundant mechanism of CD8+ T cell-mediated protection. Nevertheless, our in vivo cytolytic activity analysis also revealed significant CD95-dependent cytosis in the lung tissue and we considered the possibility that a defect in CD95-mediated cytosis could also influence CD8+ T cell-mediated protection. Unfortunately, the assessment of a combined deficiency in Pfn- and CD95-mediated killing was hindered because of technical problems. Although the transfer of WT immune CD8+ T cells into lpr mice failed to provide significant protection, lpr mice that received immune WT or Pfn-/- CD8+ T cells did not have a significant increase in Mt-specific pulmonary CD8+ T cells, suggesting a failure of T cell engagement. This lack of engagement is likely to result from increased CD95L expression on lymphoid cells in the lpr mice, leading to rejection of the CD95-expressing transferred CD8+ T cells (42). Similarly, the inability of diabetogenic T cells to transfer disease into NOD lpr mice has been linked to decreased survival of adoptively transferred T cells because of high CD95L expression in the recipients mice (43). An alternative approach might have been to obtain Mt-specific CD8+ T cells from Pfn and CD95L double-deficient mice. However, these mice have severe immune dysregulation, infertility, and die early of pancreatitis, making them unsuitable for long-term studies (44).

“Our results are the first to show that Pfn is required for Mt-specific CD8+ T cell-mediated protection against Mt infection. Despite their IFN-γ production, the transferred Pfn-/- CD8+ T cells did not afford protection to irradiated mice. This suggests that donor CD8+ T cell-derived IFN-γ is dispensable in this system, although we cannot exclude the possibility that CD8+ T cells require both IFN-γ and Pfn in a synergistic manner, perhaps even at the individual cellular level, to provide protection. Pfn-mediated cytotoxicity may help control infection by removal of a bacterial niche, by direct or indirect killing of bacteria, or by release of free bacteria for subsequent destruction by nearby phagocytic cells (13, 45). It is likely that this mechanism of host resistance is relevant for humans since CD8+ T cells express microbicidal molecules such as granulysin, which requires Pfn to kill intracellular Mt, and are present in Mt lesions of infected individuals (20, 46).

In summary, we have shown that Mt-specific CD8+ T cells utilize a variety of cytolytic mechanisms in vivo in an apparently hierarchical and overlapping manner. However, the protective effects of these various mechanisms appear less redundant, and we can conclude that the protection afforded by Mt-specific CD8+ cells is Pfn dependent. It is interesting to speculate why there is a discrepancy between the cytotoxic pathways revealed by the in vivo CTL assay and those that are protective against infection. Mt-specific T cell clones that use granule-mediated, but not CD95/CD95L, killing are associated with bactericidal activity in vitro, which may explain the specific requirement for Pfn to mediate protection in vivo (47). Although CD95-mediated apoptosis has been associated with a decline in Mt viability in vitro, this mechanism may be less efficient than Pfn-dependent killing (48).

Although peptide-pulsed splenocytes may be efficiently killed by the CD95/CD95L pathway, Mt-infected cells may be resistant to CD95L-mediated killing because of decreased CD95 expression or caspase inhibition. The identification of in vivo targets during the course of infection will further aid in understanding why Mt-specific CD8+ T cell cytotoxicity protects the host against infection and will inform the use of different vaccine strategies.

Disclosures
The authors have no financial conflict of interest.

References


