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Virally Infected Hepatocytes Are Resistant to Perforin-Dependent CTL Effector Mechanisms[1]

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Cell-mediated cytotoxicity plays an important role in the clearance of noncytopathic viruses from infected tissues. Perforin-dependent cytotoxic mechanisms have been noted to play an important role in the clearance of infections from multiple extrahepatic organs. In contrast, mice with defects in the Fas/Fas ligand (FasL)-mediated cytotoxicity pathway exhibit delayed clearance of adenovirus from the liver without apparent delay in the clearance of viral infections from extrahepatic organs. The present studies examined the role of cytotoxic effector mechanisms in intrahepatic immune responses to a replication-defective, recombinant β-galactosidase-encoding adenovirus (AdCMV-*lacZ*). Delayed clearance of AdCMV-*lacZ* from the livers of FasL-defective B6.*gld* mice, but not perforin-deficient B6.pfp$^{-/-}$ mice, was noted despite no significant differences in initial hepatic CD8$^+$ T cell IFN-γ or TNF responses or in activation of intrahepatic cytotoxic lymphocytes cells capable of killing AdCMV-*lacZ*-infected fibroblast targets. In contrast, AdCMV-*lacZ*-infected hepatocyte targets were far more sensitive to killing by intrahepatic cytotoxic lymphocytes from B6.pfp$^{-/-}$ than from B6.*gld* mice, and residual levels of virus-specific killing of hepatocyte targets by FasL-defective B6.gld CTL were blocked by TNF inhibition. These results suggest that inherent resistance of hepatocytes to cytotoxicity mediated by perforin-dependent mechanisms leaves Fas/FasL-dependent, cell-mediated cytotoxicity as the major pathway for CTL-mediated killing of virally infected hepatocytes and accounts for the more prominent role of perforin-independent anti-viral mechanisms in immune responses in the liver. ***The Journal of Immunology*****, 2001, 167: 1566–1574.**

CD8$^+$ T cell-mediated immune responses play a central role in the clearance of noncytopathic viruses from infected tissues (1, 2). These immune effector cells selectively kill virally infected cells via two major pathways mediated either by the actions of the cytolytic granule effector molecules perforin, granzyme A and granzyme B, or by the expression of cell surface Fas ligand (FasL)[3] that interacts with Fas receptors on target cells (1–3). Production of TNF-α by CD8$^+$ T cells affords an additional pathway for the induction of apoptosis (4) in virally infected cells, and CD8$^+$ T cell cytokines such as IFN-γ and TNF inhibit viral gene expression and replication by noncytopathic mechanisms (5).

Perforin-dependent cytotoxic mechanisms have been noted to play an essential role in the clearance of lymphocytic choriomeningitis virus (LCMV) infections from multiple organs (6, 7). Perforin-deficient mice also exhibit delayed clearance of mouse hepatitis virus from the CNS (8) and of CMV from the spleen (9) and salivary glands (10). In contrast, mice defective in the Fas/FasL cytotoxic effector pathway exhibit no deficiencies in the clearance of LCMV infections (11), and resolution of a variety of cytopathic viral infections proceeds normally despite deficiencies in either the perforin or the Fas/FasL cytotoxicity pathway (11). These observations have led to the conclusion that perforin-dependent cytotoxic mechanisms play a pre-eminent role in the control of noncytopathic viral infections, whereas the control of cytopathic viruses is mediated by the noncytotoxic antiviral effects of cytokines (5, 11) or granzymes (12) and/or by the production of neutralizing Abs (11).

However, in studies examining the clearance of noncytopathic, replication-defective adenoviruses from the liver, deficiencies in IFN-γ (13) or TNF (14) have been found to have more profound effects on the rates of viral clearance than deficiencies in perforin. Other studies have suggested that IFN-γ and TNF noncytopathically abolish at least 90% of hepatitis B virus replication before the induction of CTL responses in the liver (5, 15). In additional studies such cytokine effector mechanisms have been implicated in the early control of both LCMV and CMV infections of hepatocytes (8, 16), whereas cytokine effector mechanisms play lesser roles in the control of such infections in the nonparenchymal cells of liver (16) and spleen (8). Mice with deficiencies in TNF, Fas, or FasL expression exhibit delayed rates of clearance of adenoviral vectors from the liver (14, 17). In contrast, while some observers have reported delayed clearance of adenovirus from the liver of perforin-deficient mice (13), others have noted complete clearance of adenoviral vectors from the livers of perforin-deficient mice within 1 mo of infection (14). Of note, both TNF (18) and FasL (19, 20) costimulate T cell proliferative responses and adenovirus-infected TNF-deficient mice have been noted to be defective in recruiting lymphocytes to the liver (14). In addition, the infusion of inhibitors of either TNF or FasL has been reported to diminish both intrahepatic CD8$^+$ T cell infiltration and the extent of hepatocyte injury during adenovirus infection (21). Thus, it is unclear whether defects in intrahepatic adenovirus clearance in Fas/FasL-deficient mice are related predominately to defects in cell-mediated cytotoxicity or to the roles of these molecules in amplification of noncytopathic T cell antiviral mechanisms.

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[3] Abbreviations used in this paper: FasL, Fas ligand; AdCMV-*lacZ*, β-galactosidase-encoding recombinant adenovirus; TNFR-Ig, TNF receptor attached to Fc portion and hinge region of mouse IgG1 heavy chain; B6, C57BL/6; B6.*gld*, B6Smn.C3H-Fasl$^{(gld)}$; B6.pfp$^{-/-}$, C57BL/6-Pfp^{tmlSdz}; B6.TNFR1$^{-/-}$, C57BL/6-*Tnfrs1a*^{tmlMak}; LCMV, lymphocytic choriomeningitis virus; DPPI, dipeptidyl peptidase I; BLT, *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl.

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The present studies were performed to assess the role of cell-mediated, cytopathic effector mechanisms in the clearance of a replication-defective, β-galactosidase-encoding adenovirus (Ad-CMV-*lacZ*) (22) from the liver of C57BL/6 (B6) mice. Delayed clearance of AdCMV-*lacZ* from the liver of FasL-defective B6.*gld* mice, but not perforin-deficient B6.pfp$^{-/-}$ mice, was found to correlate to defects in killing of virally infected hepatocyte targets by B6.*gld*, but not by B6.pfp$^{-/-}$, CD8$^+$ T cells and could not be attributed to any defects in intrahepatic T cell cytokine responses in B6.*gld* mice. These results suggest that the absence of any apparent role for perforin-dependent cytopathic mechanisms in clearance of AdCMV-*lacZ* from the liver is related to inherent resistance of hepatocytes to cytotoxicity mediated by perforin-dependent mechanisms, which leaves Fas/FasL-dependent cell-mediated cytotoxicity as the major pathway for CTL-mediated killing of virally infected hepatocytes.

Materials and Methods

Mice

C57BL/6J (B6, H-2^b), B6Smn.C3H-Fasl$^{(gld)}$ (B6.*gld*), C57BL/6-Pfp^{tm1Sdz} (B6.pfp$^{-/-}$), B6.129-*Tnfrsf1a*^{tm1Mak} (B6.TNFR1$^{-/-}$), FVB/NJ (FVB, H-2^q), and DBA/2J (H-2^d) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice used in individual experiments were age and sex matched and were used before 12 wk of age.

Adenovirus vectors

The E1-deleted, replication-deficient, β-galactosidase-encoding recombinant adenovirus (AdCMV-*lacZ*) was propagated in 293 cell cultures and purified on cesium chloride gradient, and titers of infectious virus were determined by plaque assay as previously described (22). Mice were injected with 10^9–10^{10} PFU of AdCMV-*lacZ*. In each experiment a constant number of PFU per gram body weight was administered to each mouse. Target cells for virus-specific cytotoxicity assays were infected with Ad-CMV-*lacZ* at a multiplicity of infection of 5–10 overnight. In other experiments serum was collected from mice infected with a replication-deficient adenoviral vector encoding a chimeric fusion protein consisting of the extracellular domain of the human 55-kDa TNF receptor linked to the hinge and Fc regions of murine IgG1 (TNFR-Ig) (23). TNFR-Ig or control mouse Ig was purified from the sera by adherence to protein G-Sepharose (Amersham Pharmacia, Uppsala, Sweden) columns.

β-Galactosidase assay

β-Galactosidase activity was quantified by measuring the rate of cleavage of 4-methylumbelliferyl-β-D-galactoside to yield the fluorescent product 4-methylumbelliferone. Tissue samples were washed in PBS. The tissues were then homogenized in a buffer containing 25 mmol/L Tris-HCl (pH 7.5), 125 mmol/L NaCl, and 2 mmol/L $MgCl_2$ and centrifuged at 15,000 rpm. Then, 40 μl of the supernatant or the supernatant diluted with reaction buffer were added to the microcentrifuge tube and incubated at 37°C for 30 min with 160 μl of a reaction mixture containing 25 mmol/L Tris-HCl (pH 7.5), 125 mmol/L NaCl, 2 mmol/L $MgCl_2$, 12 mmol/L 2-mercaptoethanol, and 0.3 mmol/L 4-methylumbelliferyl-β-D-galactoside (Sigma, St. Louis, MO). The reactions were stopped by adding 50 μl 25% TCA. Tubes were cooled on ice for 5–10 min and then centrifuged at high speed for 1–2 min. Thereafter, 100 μl supernatant was added to 1.9 ml glycine-carbonate reagent. Light emission at 460 nm after excitation at 365 nm was compared with emission by standard concentrations of 4-methylumbelliferone purchased from Sigma (24).

Protein assay

Protein concentrations in tissue homogenates were assayed by the bicinchoninic acid method with reagents purchased from Pierce (Rockford, IL) and using BSA as a standard (25).

Isolation of hepatocytes and [^{3}H]thymidine labeling

Anesthetized mice underwent laparotomy, and a catheter was introduced into the inferior vena cava through the right atrium (26). The portal vein was then severed, the catheter was connected to a peristaltic pump, and the liver was perfused at 5 ml/min for 2 min with preperfusion buffer (NaCl (0.14 M), KCl (5.4 mM), Na_2HPO_4 (0.8 mM), HEPES (25 mM), EGTA (12.5 mM), sodium pyruvate (2.3 mM), L-glutamine (2.3 mM), and D-glucose (0.5 mM), pH 7.4). The liver was then perfused for 10 min with the perfusion medium (NaCl (0.14 M), KCl (5.4 mM), Na_2HPO_4 (0.8 mM), HEPES (25 mM), sodium pyruvate (2.3 mM), L-glutamine (2.3 mM), D-glucose (0.5 mM), $CaCl_2$ (2 mM), $MgSO_4$ (0.8 mM), collagenase A (0.163 U/ml; Roche, Mannheim, Germany), and DNase I (0.004%; Sigma)) (26–28). The perfused liver was removed and placed in a sterile dish containing William's medium (Life Technologies, Gaithersburg, MD) and then passed through a 100-μm pore size nylon mesh to obtain a single-cell suspension. Hepatocytes were centrifuged with 50% Percoll (Amersham) at 500 rpm for 15 min to separate dead cells, then cultured in plates coated with 1% collagen type I (Sigma) in William's medium supplemented with FBS (10%), HEPES (25 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), fungisone (5 μg/ml), insulin (10 μg/ml), transferrin (10 μg/ml), selenous acid (10 ng/ml), dexamethasone (1 μM), epidermal growth factor (5 nM/ml), glucagon (0.1 μM), somatotropin (10 μU/ml), and prolactin (20 mU/ml; all purchased from Sigma) and added to the medium immediately before use. For [^{3}H]thymidine labeling, the cells were incubated for 24–48 h in the same medium with 5–10 μCi/ml [^{3}H]thymidine, recombinant human hepatocyte growth factor (15 ng/ml; Calbiochem, La Jolla, CA) and norepinephrine (10^{-5} M; Sigma) (29).

Isolation of hepatic lymphocytes

Mice were heparinized with an i.p. injection of 10 U heparin. After CO_2 narcosis, the abdomen was entered under sterile technique, the portal vein was cut, and the abdominal portion of the vena cava was perfused with 20 ml Ca^{2+}- and Mg^{2+}-free phosphate buffer solution preheated to 37°C. The liver was removed and passed through a 40 mesh pore size screen, then a 300 mesh pore size screen (VWR scientific, catalogue no. EC587–40 and EC589–30, respectively) (30, 31). The cell suspension was centrifuged with 35% Percoll at 1500 rpm for 15 min, and the cell pellet was cultured in a 75-cm^2 flask in complete medium supplemented with 10% FBS (Life Technologies), 100 U/ml penicillin, 1 μg/ml gentamicin, and 2 mM L-glutamine (Sigma), for 4 h. Then, the lymphocytes were aspirated and centrifuged again with 35% Percoll. Afterward, cells were used as effectors in cytotoxicity assays or for flow cytometric studies.

CD8$^+$ T cell purification

CD8$^+$ intrahepatic lymphocytes were purified by positive selection using magnetic cell sorting with a VarioMACS cell sorter (Miltenyi Biotec, Auburn, CA) (32). Briefly, cells were suspended in PBS buffer supplemented with 5 mM EDTA and 1% FBS and incubated for 15 min at 4°C with anti-CD8$^+$ Ab-coated microbeads. Positive selection columns were used, and cell populations were washed with PBS following separation and isolation as directed by the manufacturer. Upon a subsequent flow cytometric analysis of MACS-purified CD8$^+$ T lymphocytes, >95% purity of CD8$^+$ T cells was observed.

Flow cytometric analysis

Spleen cells, hepatic lymphocytes, or cultured cell lines were washed and incubated for 30 min at 4°C with FITC-labeled anti-CD4 (L3T4, GK1.5), anti-CD8a (Ly-2, 53-6.7), anti-TCRαβ (H57-597), anti-TCRγδ (GL3), anti-NK1.1 (DX5), anti-H-2K^q (KH114), or the appropriate isotype control. In other experiments cells were washed and incubated for 30 min at 4°C with unconjugated anti-H-2K^b (Y-3) or anti-H-2D^q (28-14-8s) or the appropriate isotype control and then washed and incubated for an additional 20 min at 4°C with FITC-labeled goat anti-mouse IgG. All Abs used were purchased from BD PharMingen (San Diego, CA) or were produced as culture supernatant of hybridomas purchased from American Type Culture Collection (Manassas, VA). The cells were analyzed by fluorescence-activated flow cytometry on a FACScan. For assessment of intracellular IFN-γ and TNF expression, cells were incubated in 5% CO_2, 37°C incubator with PMA (50 ng/ml), A23187 (500 ng/ml), and brefeldin A (10 μg/ml) for 4 h. Cells were labeled with the surface-staining Abs as mentioned above, and then fixed with 4% formaldehyde at room temperature for 10 min. After fixation, the cells were incubated on ice for 1 h with saponin-containing medium to permeabilize the membranes. The PE-labeled anti-IFN-γ (XMG1.2) or PE-labeled anti-TNF (MP6-XT22) Abs were added and incubated at 4°C for 1 h. This was followed by two washes with saponin-containing medium and one final wash with normal staining medium (33). The cells were analyzed by fluorescence-activated flow cytometry on a FACScan.

Cell lines

The Hepa-1 hepatoma cell line (H-2^b), the nontransformed AML-12 hepatocyte cell line, the P815 mastocytoma cell line (H-2^d), as well as the

BLK-cl4 (H-2^b) and 3T3 fibroblast cell lines were purchased from American Type Culture Collection. The H-2 phenotypes of AML-12 hepatocytes and 3T3 fibroblasts were ascertained by flow cytometric analysis using a panel of allele-specific anti-H-2K or anti-H-2D mAb with AML-12 cells found to be anti-H-$2K^b$ and anti- H-$2D^q$ positive, while 3T3 fibroblasts were found to be anti-H-$2K^q$ and anti-H-$2D^q$ positive.

Dipeptidyl peptidase I (DPPI) and N-α-*benzyloxy-carbonyl-*L*-lysine thiobenzyl (BLT) esterase assays*

DPPI activity was assayed by the hydrolysis of glycylphenylalanyl-β-naphthylamide (100 μM) in 600 μl of 50 mM sodium acetate-acetic acid (pH 5.5), 30 mM NaCl, 1 mM DTT, and 0.5 mM EDTA as previously described (34). After incubation for 20 min at 37°C, the reaction was stopped by the addition of 500 μl of 50 mM glycine-NaOH, pH 10.5. Substrate hydrolysis was monitored by the fluorescence of β-naphthylamine released per minute. BLT esterase activity was assessed in an assay mixture (200 μl) containing 25–100 μl homogenized cells supernatant (1 million cells) and substrate mix composed of 120 μl of 0.2 mM BLT (Calbiochem-Behring, La Jolla, CA), 0.22 mM 5,5′dithio-bis-(2)-nitrobenzoic acid (Sigma), and 0.2 M Tris-HCl in sodium PBS as previously described (35). The mixture was incubated at 37°C, and the change in absorbance at 410 nm was measured compared with that in samples containing no cell extract.

Generation of allospecific CTL

In vitro-activated allospecific CTL were generated in 5-day MLC containing 10–12 million responder spleen cells from B6 mice and an equal number of irradiated FVB or DBA/2J spleen cells to generate anti H-2^q- and H-2^d-specific CTL, respectively (36).

Chromium release assay

Targets were labeled with 150 μCi Na_2CrO_4 for 60–90 min at 37°C and were washed twice before incubation with the different effectors at different E:T cell ratios in 200-μl cultures. After 12 or 18 h, 100 μl supernatant was harvested from experimental and control wells, and the percent specific lysis as calculated from the formula: % specific lysis = [(experimental release (cpm) − spontaneous release (cpm))/(maximal release (cpm) − spontaneous release (cpm)] × 100. Virus-specific cytotoxicity was calculated by subtracting the percent specific lysis of noninfected targets from the percent specific lysis of infected targets (36). All assays were performed in triplicate, and the results are shown as the mean ± SEM.

JAM test for apoptosis (DNA fragmentation)

[^{3}H]thymidine was added to culture dishes for 12–18 h at a final concentration of 2.5–5 μCi/ml. Targets were harvested and cultured with effector cells as in the chromium release assay. At the end of the assay cells were aspirated onto fiberglass filters by vacuum suction, DNA was dried, and [^{3}H]thymidine-labeled high m.w. DNA was quantitated by scintillation counting. The specific DNA fragmentation was determined using the following formula: % specific DNA fragmentation = [(control (cpm) − experimental (cpm))/control (cpm)] × 100, with control cpm being the total [^{3}H]thymidine retained in the absence of effector cells (37). In some assays 1 μg protein G-Sepharose-purified TNFR-Ig or control Ig was added to each well of the cytotoxicity assays.

Results

*Delayed clearance of AdCMV-*lacZ *from liver of Fas-L-defective mice*

FasL-defective B6.*gld*, perforin-deficient B6.pfp$^{-/-}$ and control B6 mice were infected with AdCMV-*lacZ* and sacrificed at different time points to assess hepatic expression of the adenoviral transgene product, β-galactosidase. As shown by the results of two experiments detailed in Fig. 1, similar levels of β-galactosidase expression were observed in the livers of all three strains of mice during the first 10 days after AdCMV-*lacZ* infection. Whereas β-galactosidase expression declined over a similar time course in B6 and B6.pfp$^{-/-}$ mice, significantly higher levels of the AdCMV-*lacZ* transgene product were detected in livers of B6.*gld* mice at days 21, 30, and 42 after adenoviral infection (Fig. 1).

*Similar expansion of CD8$^+$ and CD4$^+$ T cells in liver of AdCMV-*lacZ*-infected B6, B6.*gld*, and B6.pfp$^{-/-}$ mice*

To assess intrahepatic antiviral T cell responses, intrahepatic lymphocytes were isolated, counted, and stained with anti-CD4, anti-CD8, anti-TCRαβ, anti-TCRγδ, anti-NK1.1, and anti-Ig Abs 7 days after AdCMV-*lacZ* infection. Flow cytometric analysis of intrahepatic lymphocytes revealed a 10- to 15-fold increase in the total number of intrahepatic lymphocytes in all strains of mice (Fig. 2, *left*) following AdCMV-*lacZ* infection. In adenovirally infected mice 65–70% of intrahepatic lymphocytes expressed TCRαβ, indicating that the majority of lymphocytes infiltrating the liver were T cells, while only modest increases in the numbers of intrahepatic Ig$^+$ B cells (10–15% of total cells) and TCRγδ$^+$ T cells (6–8%) and no changes in the absolute numbers of NK1.1$^+$ intrahepatic NK cells were detected 7 days after AdCMV-*lacZ* infection. As detailed in Fig. 2 (*middle* and *right*), the absolute numbers of CD8$^+$ and CD4$^+$ T cells detected in livers of B6, B6.*gld*, and B6.pfp$^{-/-}$ mice 7 days after AdCMV-*lacZ* infection were not significantly different, with severalfold greater expansion of CD8$^+$ vs CD4$^+$ liver T cells noted in each mouse strain.

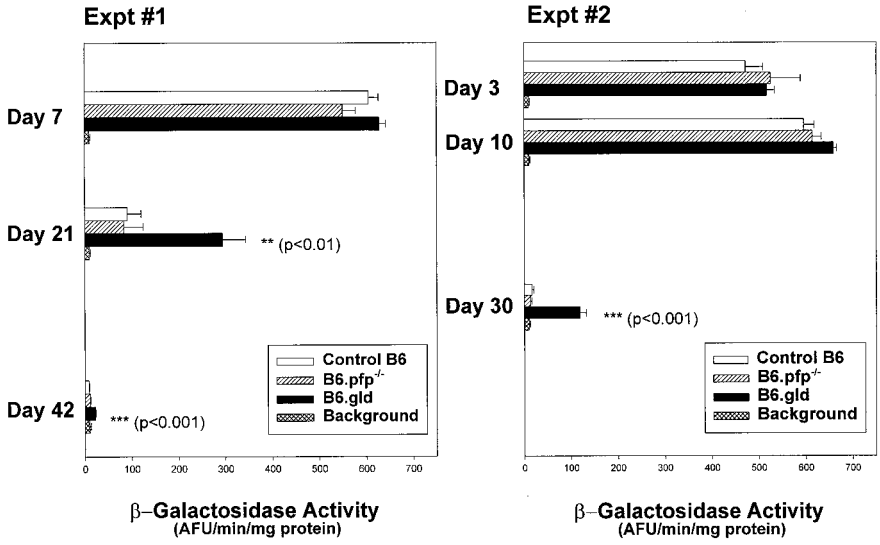

FIGURE 1. Clearance of adenovirus from liver of B6.*gld* (Fas-L defective), B6.pfp$^{-/-}$ (perforin-deficient), and control B6 mice. Three or four mice per experimental group were infected with AdCMV-*lacZ*, and β-galactosidase expression in the liver was assessed at the indicated time points.

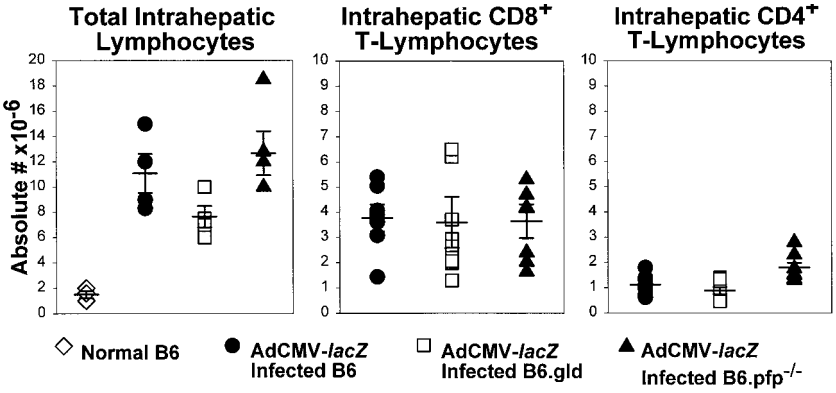

FIGURE 2. Increase in number of intrahepatic lymphocytes following AdCMV-*lacZ* infection. *Left*, Total numbers of intrahepatic lymphocytes isolated from normal B6 (◇), AdCMV-*lacZ*-infected B6 (●), B6.*gld* (□), and B6.pfp$^{-/-}$ (▲) mice; *middle* and *right*, absolute numbers of liver CD8$^+$ and CD4$^+$ T cells, as determined by flow cytometric analysis. Results are the mean ± SEM of three to five mice from each experimental group.

Intracellular expression of IFN-γ and TNF in splenic and intrahepatic CD8$^+$ and CD4$^+$ lymphocytes

In subsequent experiments spleen and liver lymphocytes from AdCMV-*lacZ*-infected mice were assessed for cytokine responses. As shown by the results of a representative experiment detailed in Fig. 3, the highest level of IFN-γ expression was detected in liver CD8$^+$ T cells, although a smaller fraction of liver CD8$^-$ lymphocytes and spleen CD8$^+$ or CD8$^-$ T lymphocytes also expressed IFN-γ. As detailed in Fig. 4, similar numbers of liver CD8$^+$ and CD4$^+$ T cells from adenovirally infected B6, B6.*gld* and B6.pfp$^{-/-}$ mice expressed IFN-γ and TNF, indicating no discernible differences in the intrahepatic cytokine responses to AdCMV-*lacZ* in these three strains of mice.

*Virus-specific killing of AML12 targets by intrahepatic lymphocytes from AdCMV-*lacZ*-infected mice*

As B6.*gld* and B6.pfp$^{-/-}$ mice exhibited no deficiencies or any compensatory increases in intrahepatic T cell cytokine responses to viral infection, additional studies were designed to assess the activation of CTL effector function in the livers of AdCMV-*lacZ*-infected mice. In initial experiments AML12 cells, a line of nontransformed hepatocytes that has been noted to maintain a differentiated hepatocyte phenotype during long term culture (38), were used as targets in CTL assays of varying duration. No killing of AdCMV-*lacZ*-infected AML12 cells by in vivo activated intrahepatic lymphocytes from B6, B6.*gld*, or B6.pfp$^{-/-}$ mice was detected in assays of ≤4-h duration (data not shown). However, as illustrated by the results of a representative experiment detailed in Fig. 5, in longer 18-h assays virus-specific killing of AdCMV-*lacZ*-infected AML12 cells by in vivo-activated intrahepatic lymphocytes isolated from B6.pfp$^{-/-}$ mice 7 days after AdCMV-*lacZ* infection was readily detected, whereas intrahepatic lymphocytes isolated from AdCMV-*lacZ*-infected B6.*gld* mice mediated no virus-specific killing of AML12 cells. These results indicated that AML12 cells are susceptible to the FasL-mediated cytotoxicity mechanisms previously reported to represent the major cytotoxicity pathways mediated by CTL from perforin-deficient mice (3) and defective in B6.*gld* CTL. In addition, these results suggested that either AML12 cells are resistant to the perforin- and granzyme-mediated cytotoxicity pathway that is preserved in B6.*gld* CTL or that this effector pathway was not activated in intrahepatic B6.*gld* lymphocytes during AdCMV-*lacZ* infection.

DPPI and granzyme A activity in CD8$^+$ intrahepatic T lymphocytes

Additional studies were performed to assess the levels of CTL granule effector molecules in B6.*gld* mice. While reagents for quantitative assessment of murine perforin protein expression in

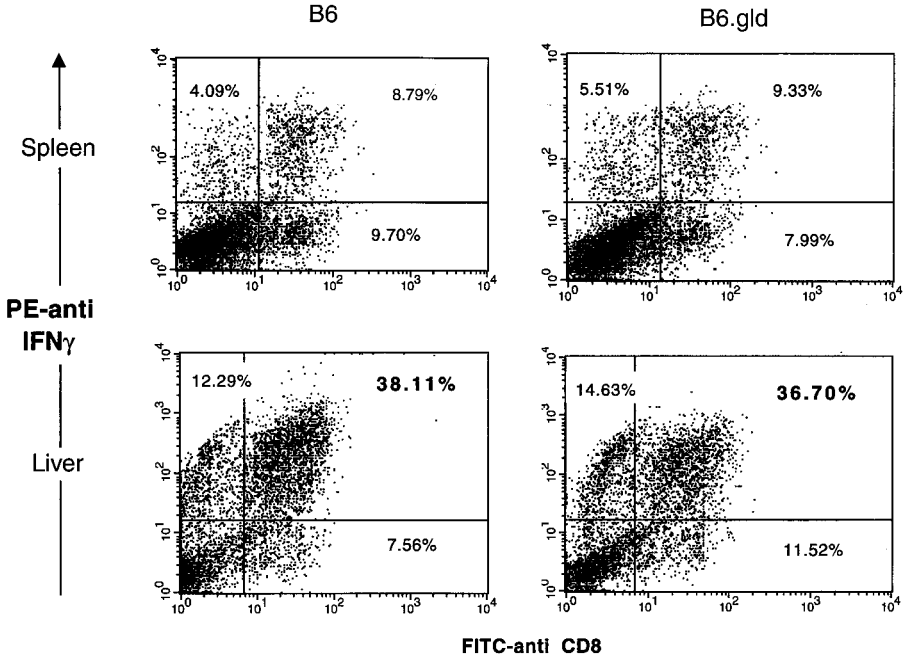

FIGURE 3. Intracellular expression of IFN-γ in spleen and liver T cells. B6 and B6.*gld* splenocytes and intrahepatic lymphocytes obtained 7 days after AdCMV-*lacZ* infection were double stained with FITC-anti-CD8 and PE-anti-IFN-γ as detailed in *Materials and Methods*.

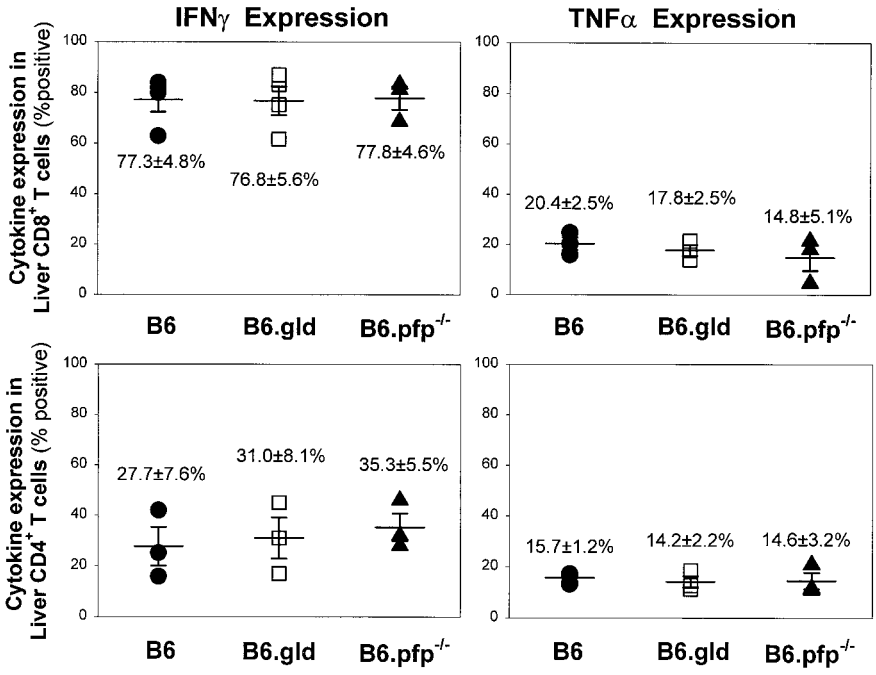

FIGURE 4. Cytokine expression in liver CD8$^+$ T cells following AdCMV-*lacZ* infection. Intrahepatic lymphocytes were isolated from B6 (●), B6.*gld* (□), and B6.pfp$^{-/-}$ (▲) mice 7 days after infection with AdCMV-*lacZ* and double stained with anti-CD8 and anti-IFNγ or anti-TNF as detailed in *Materials and Methods*. Results are shown from three or four staining experiments as the mean ± SEM.

CD8$^+$ T cells were not available, sensitive quantitative assays have been used to assess levels of the granule proteases granzyme A and DPPI that are coexpressed with perforin in CTL effector granules (39). In the experiment detailed in Fig. 6, levels of granzyme A (BLT esterase) and DPPI activities were assayed in purified CD8$^+$ lymphocytes extracted from livers of B6 and B6.*gld* mice 7 days after infection with AdCMV-*lacZ*. CD8$^+$ intrahepatic lymphocytes of noninfected B6 mice served as controls. The levels of expression of the CTL granule proteases DPPI and granzyme A in infected B6 and infected B6.*gld* mice were similar and dramatically higher than the levels of expression detected in control intrahepatic CD8$^+$ T cells.

*Virus-specific killing by spleen and liver lymphocytes from AdCMV-*lacZ*-infected mice*

To further assess anti-viral CTL responses in B6.*gld* mice, lymphocytes were isolated from spleens and livers of B6.*gld* and B6.pfp$^{-/-}$ mice 1 wk after infection with AdCMV-*lacZ* and assessed for adenovirus-specific killing of Hepa-1 hepatoma and control BLK-cl4 fibroblast targets. In the experiments detailed in Fig. 7, splenic and intrahepatic lymphocytes from adenovirally infected B6.*gld* and B6.pfp$^{-/-}$ were equally effective in killing AdCMV-*lacZ*-infected BLK-cl4 fibroblast targets (Fig. 7, *B* and *D*). In contrast, B6.*gld* CTL were found to mediate significantly reduced levels of killing of AdCMV-*lacZ*-infected hepa-1 targets compared with the much higher levels of killing of these targets by splenic and intrahepatic lymphocytes isolated from AdCMV-*lacZ*-infected B6.pfp$^{-/-}$ (Fig. 7, *A* and *C*) mice. These results indicated that cytolytic activity capable of killing virally infected fibroblast targets was indeed activated in both spleen and liver of B6.*gld* mice during AdCMV-*lacZ* infection. However, targets of hepatocyte origin were again found to be relatively resistant to FasL-independent effector mechanisms.

To better assess CTL mechanisms capable of killing hepatocytes, additional experiments were designed to measure killing of AdCMV-*lacZ*-infected hepatocytes isolated directly from B6 mice. Similar to results obtained with AML-12 target cells, no killing of AdCMV-*lacZ*-infected B6 hepatocytes by intrahepatic lymphocytes from AdCMV-*lacZ*-infected B6 mice was observed in cytotoxicity assays of ≤4-h duration (data not shown). In contrast, as illustrated by the results of the representative experiment detailed in Fig. 8*A*, virus-specific killing of B6 hepatocytes targets by intrahepatic B6.CTL was observed in 18-h DNA fragmentation assays, indicating that CTL effector mechanisms with slower kinetics than typically observed with granule-mediated cytotoxicity were probably responsible for inducing hepatocyte apoptosis. In addition, intrahepatic lymphocytes from B6.*gld* mice were much less efficient in killing B6 hepatocytes than control B6 CTL (Fig. 8*A*). To elucidate the CTL effector mechanisms responsible for the

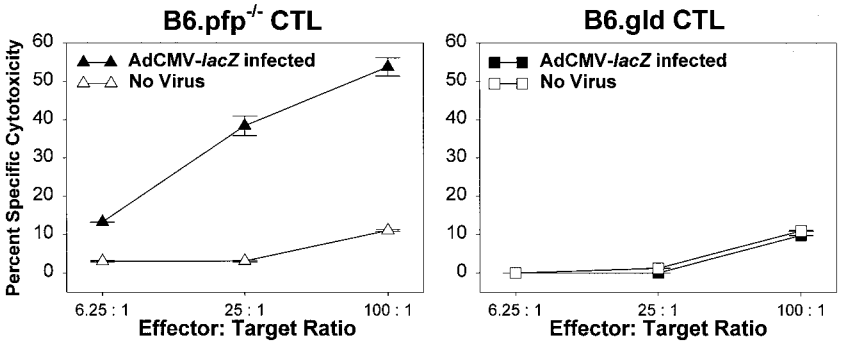

FIGURE 5. Virus-specific killing of AdCMV-*lacZ*-infected AML12 hepatocyte targets by in vivo-activated intrahepatic lymphocytes. Lymphocytes were extracted from the livers of B6.*gld* (□) and B6.pfp$^{-/-}$ (▲) mice 7 days after AdCMV-*lacZ* infection and were assessed for the ability to kill AdCMV-*lacZ*-infected or control AML12 hepatocyte targets at the indicated E:T cell ratios in a 12-h assay.

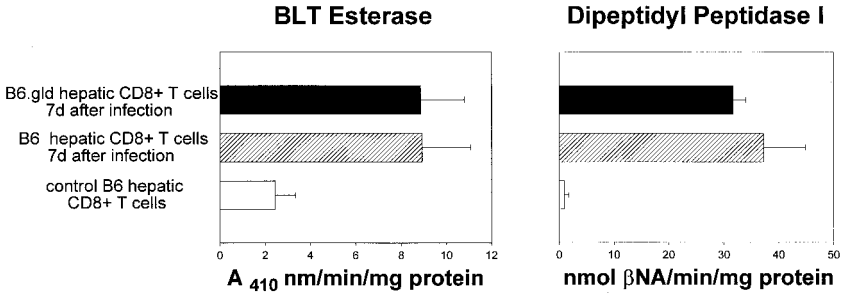

FIGURE 6. Expression of DPPI and granzyme A (BLT esterase) activities in $CD8^+$ hepatic lymphocytes. Lymphocytes were isolated from livers of noninfected B6, AdCMV-*lacZ*-infected B6, and AdCMV-*lacZ*-infected B6.*gld* mice 7 days after the infection. $CD8^+$ T cells were separated by MACS using anti-CD8 magnetic beads as detailed in *Materials and Methods* and were assayed for DPPI or BLT esterase activity within whole cell lysates. Results are the mean ± SEM of five separate experiments.

modest levels of hepatocyte killing mediated by B6.*gld* CTL, additional experiments were performed using hepatocyte targets deficient in type I TNFR (Fig. 8*B*) or a recombinant TNFR-Ig fusion protein previously found to very efficiently neutralize TNF (Fig. 8, *C* and *D*). As illustrated in Fig. 8, the disparity between B6 and B6.*gld* CTL effector function was even more dramatic when $TNFR1^{-/-}$ hepatocyte targets were employed (Fig. 8*B*), and addition of a TNF inhibitor to cytotoxicity assays partially inhibited B6 CTL killing of AdCMV-*lacZ*-infected hepatocytes (Fig. 8*C*) and blocked all killing of these targets by B6.*gld* CTL (Fig. 8*D*). Thus, in contrast to BLK-cl4 fibroblast targets, killing of hepatocyte targets appeared to be mediated predominantly by Fas/FasL- and TNF/TNFR1-dependent mechanisms.

Allospecific killing by splenocytes

To determine whether the results of experiments detailed in Figs. 6–8 were related to an intrinsic resistance of hepatic targets cells to killing by the perforin- and granzyme-dependent CTL effector pathway or to a selective resistance of adenovirus-infected hepatocytes to this killing mechanism, the experiments detailed in Fig. 9 were performed. Spleen cells were isolated from B6, $B6.pfp^{-/-}$, and B6.*gld* and then stimulated in vitro for 5 days with irradiated splenocytes from FVB ($H\text{-}2^q$) mice or DBA/2J ($H\text{-}2^d$) mice to generate allospecific CTL. The FVB-stimulated lymphocytes were used to kill $H\text{-}2D^q$-expressing AML12 hepatocytes and $H\text{-}2K^qD^q$-expressing 3T3 fibroblasts, whereas DBA/2J-stimulated lymphocytes were assessed for killing of $H\text{-}2^d$-expressing P815 mastocytoma cells. AML12 hepatocytes were susceptible to killing by B6 and $B6.pfp^{-/-}$ effector cells, while B6.*gld* effector cells mediated significantly less killing of this target (Fig. 9, *left*). The same populations of B6, $B6.pfp^{-/-}$, and B6.*gld* allospecific CTL mediated equivalent levels of killing of 3T3 fibroblast targets. In contrast, $B6.pfp^{-/-}$ CTL were strikingly less effective than B6 or B6.*gld* CTL in killing P815 mastocytoma targets.

Discussion

FasL-mediated killing of target cells by $CD8^+$ T effector cells was initially identified as an alternative, apparently secondary, cytotoxicity pathway that is most readily detected under conditions in which perforin-dependent cytotoxicity is impaired (3). The observation that defects in perforin-dependent CTL activity, but not in Fas/FasL interactions delay the clearance of LCMV infections led to the suggestion that perforin-dependent mechanisms are the dominant cytopathic effector pathways responsible for the clearance of noncytopathic viral infections (6, 7, 11). However, the results of the present studies as well as a series of prior observations (14, 17, 21, 40, 41) indicate that FasL-mediated immune effector mechanisms play a more important role in immune responses to noncytopathic viral infections in the liver than previously noted in viral infections in other organs. The more important role of Fas/FasL-dependent immune effector mechanisms in the liver has been attributed to the fact that hepatocytes constitutively express Fas and readily undergo apoptosis when Fas receptors are engaged by anti-Fas or FasL (42, 43). However, the results of the present studies indicate that hepatocyte-derived target cells are inherently resistant to CTL killing mediated by the perforin- and granzyme-dependent granule exocytosis pathway. These results suggest that it is the absence of perforin-mediated cytotoxicity in the liver that results in a greater dependence upon FasL-dependent cytotoxicity and other perforin-independent antiviral mechanisms in the clearance of viral pathogens from the liver.

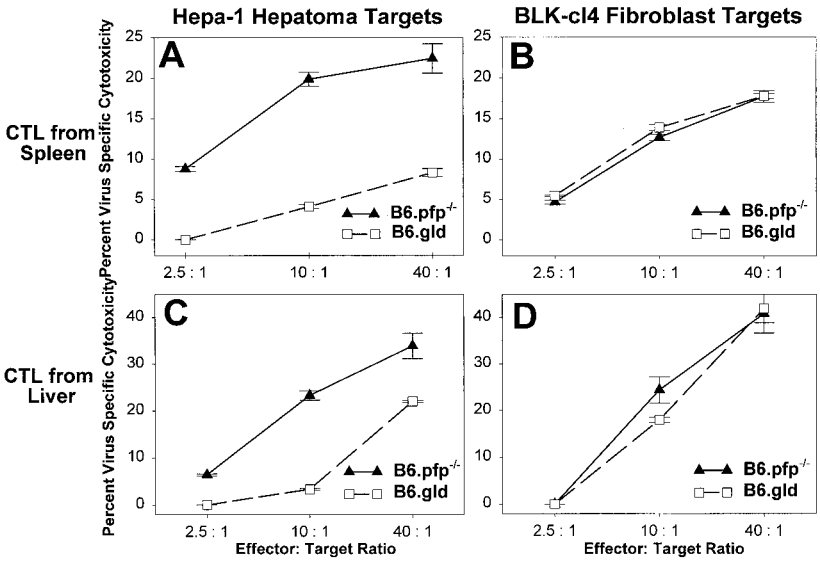

FIGURE 7. Virus-specific killing of AdCMV-*lacZ*-infected hepatoma and fibroblast targets by in vivo-activated spleen and liver lymphocytes. Lymphocytes were extracted from spleens and livers of $B6.pfp^{-/-}$ (▲) and B6.*gld* (□) mice 7 days after infection with AdCMV-*lacZ* and were assessed for killing of AdCMV-*lacZ*-infected or control Hepa-1 hepatoma or BLK-cl4 fibroblast targets in an 18-h assay. Virus-specific killing was determined by subtracting the background percent specific lysis of noninfected control targets from that of infected targets. Data are representative results of one of three similar experiments.

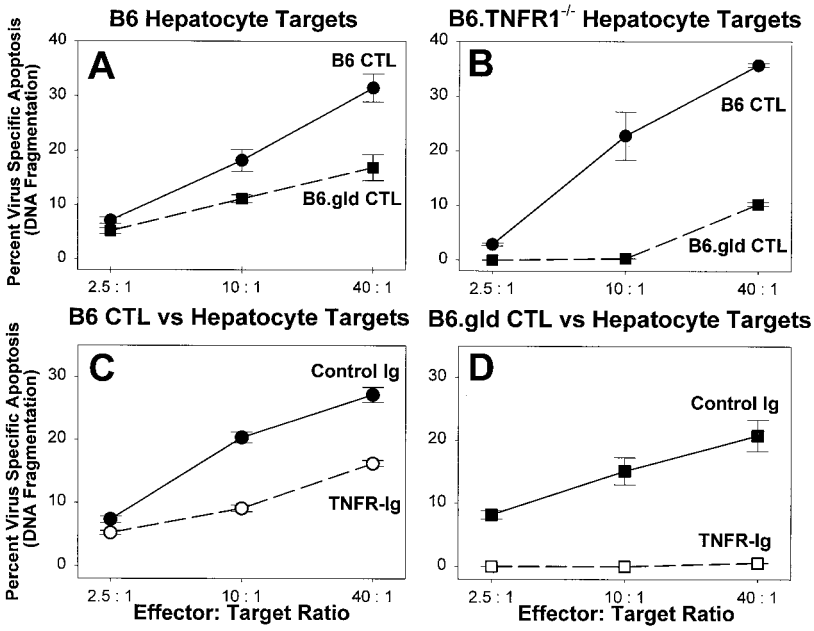

FIGURE 8. Virus-specific killing of AdCMV-*lacZ*-infected B6 and B6. TNFR1$^{-/-}$ hepatocyte targets. Intrahepatic lymphocytes isolated from B6.*gld* (□) and B6 (●) livers 7 days after infection with AdCMV-*lacZ* were assessed for the ability to kill control or AdCMV-*lacZ*-infected B6 (*A*, *C*, and *D*) or B6.TNFR1$^{-/-}$ hepatocytes (*B*) in 18-h assays. Virus-specific killing was determined by subtracting the background percent killing of noninfected control targets from that of infected targets. Results detailed in all four panels represent killing mediated by the same populations of effector cells and are representative of three separate experiments assessing killing of AdCMV-*lacZ*-infected hepatocytes by B6 and B6.*gld* intrahepatic lymphocytes.

In addition to a putative role in cytopathic effector mechanisms directed against virally infected cells, Fas/FasL interactions have been noted to play a major role in immunoregulation. Mice with the *lpr* and *gld* mutations in Fas and FasL, respectively, develop autoimmunity and lymphoproliferative syndromes (44), and FasL-defective *gld* CD4$^+$ T cells have been reported to exhibit augmented proliferative responses, while FasL-defective *gld* CD8$^+$ T cells exhibit depressed proliferative responses to antigenic stimulation (19, 20). Other investigators have reported diminished intrahepatic T cell infiltrates in livers of adenovirally infected *gld* mice (21). However, in the course of the present studies *gld* mice with defective FasL function did not exhibit any decrease or other apparent abnormality in intrahepatic CD4$^+$ or CD8$^+$ T cell proliferation or cytokine responses. In addition, intrahepatic CD8$^+$ T cells from AdCMV-*lacZ*-infected *gld* mice exhibited levels of granule proteases comparable to those of CD8$^+$ T cells from AdCMV-*lacZ*-infected B6 control mice and readily killed AdCMV-*lacZ*-infected fibroblasts. These findings indicate that both antiviral cytokine responses and granule exocytosis-mediated cytotoxicity mechanisms were induced at normal levels in the intrahepatic CD8$^+$ T cells of AdCMV-*lacZ*-infected *gld* mice in the current studies. While the explanation for the differences between the present results and those of other investigators who have reported defects in CD8$^+$ T proliferative responses in FasL-defective mice (19–21) remains unclear, it should be noted that in the present experiments mice were used at a young age before the development of immunodeficiencies more apparent in aged *gld* mice (44), and immune responses in these young *gld* mice were only assessed over the relatively brief time course during which adenoviral vectors are cleared from immunocompetent mice. Indeed, the only overt defect in intrahepatic T cells from AdCMV-*lacZ*-infected *gld* mice noted in the present studies was an inability to kill AdCMV-*lacZ*-infected hepatocyte or hepatoma targets at levels comparable to those of effector cells from control or B6.pfp$^{-/-}$ mice. Thus, the present findings indicate that rapid clearance of AdCMV-*lacZ* from the liver is dependent upon FasL-mediated cytotoxicity.

The resistance of AdCMV-*lacZ*-infected hepatocytes to killing by the perforin- and granzyme-dependent granule exocytosis effector mechanisms that remain intact in *gld* T cells cannot be attributed solely to the induction of previously reported immune diversionary mechanisms induced by the adenoviruses (45), since in the present studies uninfected hepatocyte cell line targets were equally resistant to allospecific *gld* CTL. Rather, hepatocytes appear to be inherently resistant to this cytopathic effector mechanism. The functional importance of this resistance of hepatocytes to perforin-dependent cytotoxicity is illustrated by the fact that no delay in the clearance of intrahepatic, adenovirally encoded β-galactosidase was noted in perforin-deficient mice despite measurement of this viral marker at multiple time points after adenovirus infusion. The present findings are in agreement with another report examining adenovirus-encoded transgene expression at a single time point 1 mo after

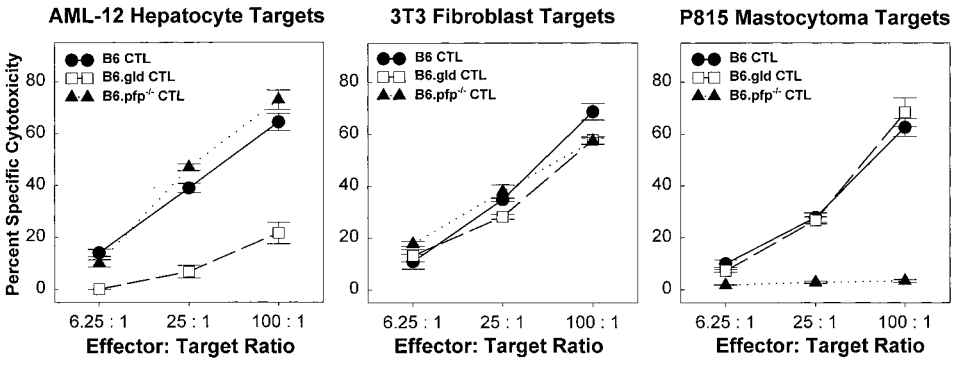

FIGURE 9. CTL killing of allospecific hepatocyte and nonhepatic targets. B6 (●), B6.*gld* (□), and B6.pfp$^{-/-}$ (▲) effector cells were generated in 5-day MLC with stimulator cells from FVB (H-2^q) mice (*left* and *middle*) or DBA/2J (H-2^d) mice (*right*) and assessed for killing of AML-12 and 3T3 targets in 18-h assays and for killing of P815 targets in 5-h assays. Data represent one of three experiments with similar results.

adenovirus infusion (14) and with the results of studies examining intrahepatic immune responses to murine CMV infection in perforin-deficient mice (8). While the mechanisms underlying hepatocyte resistance to perforin-dependent CTL effector mechanisms remain unclear, previous reports have noted similar resistance to the granule exocytosis-mediated pathway of cytotoxicity in target cells that fail to bind perforin (46) or granzyme B (47) or that express high levels of *bcl-2* (48) or the serpin proteinase inhibitor 9 that functionally blocks granzyme B activity (49).

While clearance of AdCMV-*lacZ*-encoded gene products was significantly delayed in B6.*gld* mice, the level of the transgene protein product β-galactosidase in livers of B6.*gld* mice decreased over time to nearly undetectable levels 7 wk after infection. Of note, the modest levels of killing of adenovirally infected hepatocytes that were found to be mediated by B6.*gld* CTL were further reduced or abolished if TNFR1-deficient targets were employed or TNF actions were blocked by addition of a soluble TNF inhibitor to cytotoxicity assays. These results suggest that TNF-mediated cytotoxicity may account for the clearance of AdCMV-*lacZ*-infected hepatocytes from B6.*gld* mice. Alternatively, it is possible that noncytopathic antiviral effects of $CD8^+$ T cell cytokines such as IFN-γ and TNF were responsible for eventual ablation of AdCMV-*lacZ* infection and transgene expression in these FasL-defective mice. Indeed, since many of the antiviral mechanisms mediated by cytokines are directed toward inhibiting viral proliferation, the use of a replication-deficient virus in these studies probably diminished the relative importance of the noncytopathic cytokine mechanisms that other investigators have found to be of importance in limiting viral replication and facilitating eventual immune clearance of hepatic viral infections (5, 8, 15, 16). Nevertheless, the significant delay in ablation of hepatic viral gene products in FasL-defective B6.*gld* mice despite the high frequency of cytokine-expressing intrahepatic T cells argues that cytopathic mechanisms play a role in efficient clearance of hepatic viral infections. Furthermore, the inherent resistance of hepatocytes to granule exocytosis-mediated cytotoxicity probably accounts for the increased dependence upon FasL-dependent cytotoxicity and cytokine-mediated antiviral mechanisms during immune responses to hepatic viral infections.

Acknowledgments

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References

1. Zinkernagel, R. M. 1996. Immunology taught by viruses. *Science 271:173.*
2. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science 272:54.*
3. Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science 265:528.*
4. Braun, M. Y., B. Lowin, L. French, H. Acha-Orbea, and J. Tschopp. 1996. Cytotoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. *J. Exp. Med. 183:657.*
5. Guidotti, L. G., T. Ishikawa, M. W. Hobbs, B. Matzke, R. Schreiber, and F. V. Chisari. 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity 4:25.*
6. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Oslen, E. R. Podack, R. M. Zinkernagel, and H. Hentgartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature 369:31.*
7. Walsh, C. M., M. Matloubian, C. C. Liu, R. Ueda, C. Kurahara, J. L. Christensen, M. T. F. Huang, J. D. E. Young, R. Ahmed and W. R. Clark. 1994. Immune function in mice lacking the perforin gene. *Proc. Natl. Acad. Sci. USA 91:10854.*
8. Lin, M. T., S. A. Stohlman, and D. R. Hinton. 1997. Mouse hepatitis virus is cleared from the central nervous systems of mice lacking perforin-mediated cytolysis. *J. Virol. 71:383.*
9. Tay, C. H., and R. M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J. Virol. 71: 267.*
10. Riera, L., M. Gariglio, G. Valente, A. Mullbacher, C. Museteanu, S. Landolfo, and M. M. Simon. 2000. Murine cytomegalovirus replication in salivary glands is controlled by both perforin and granzymes during acute infection. *Eur. J. Immunol. 30:1350.*
11. Kagi, D., P. Seiler, J. Pavlovic, B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1995. The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur. J. Immunol. 25: 3256.*
12. Mullbacher, A., K. Ebnet, R. V. Blanden, R. T. Hla, T. Stehle, C. Museteanu, and M. M. Simon. 1996. Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. *Proc. Natl. Acad. Sci. USA 93:5783.*
13. Yang, Y., Z. Xiang, H. C. Ertl, and J. M. Wilson. 1995. Upregulation of class I major histocompatibility complex antigens by interferon γ is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. *Proc. Natl. Acad. Sci. USA 92:7257.*
14. Elkon, K. B., C. C. Liu, J. G. Gall, J. Trevejo, M. W. Marino, K. A. Abrahamsen, X. Song, J. L. Zhou, L. J. Old, R. G. Crystal, et al. 1997. Tumor necrosis factor alpha plays a central role in immune-mediated clearance of adenoviral vectors. *Proc. Natl. Acad. Sci. USA 94:9814.*
15. Guidotti, L. G., and F. V. Chisari. 2000. Cytokine-mediated control of viral infections. *Virology 273:221.*
16. Guidotti, L. G., P. Borrow, A. Brown, H. McClary, R. Koch, and F. V. Chisari. 1999. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. *J. Exp. Med. 189:1555.*
17. Chirmule, N., A. D. Moscioni, Y. Qian, R. Qian, Y. Chen, and J. M. Wilson. 1999. Fas-Fas ligand interactions play a major role in effector functions of cytotoxic T lymphocytes after adenovirus vector-mediated gene transfer. *Hum. Gene Ther. 10:259.*
18. Brown, G. R., and D. L. Thiele. 2000. Enhancement of MHC class I-stimulated alloresponses by TNF/TNF receptor (TNFR)1 interactions and of MHC class II-stimulated alloresponses by TNF/TNFR2 interactions. *Eur. J. Immunol. 30: 2900.*
19. Suzuki, I., and P. J. Fink. 2000. The dual functions of Fas ligand in the regulation of peripheral $CD8^+$ and $CD4^+$ T cells. *Proc. Natl. Acad. Sci. USA 97:1707.*
20. Suzuki, I., S. Martin, T. E. Boursalian, C. Beers, and P. J. Fink. 2000. Fas ligand costimulates the in vivo proliferation of $CD8^+$ T cells. *J. Immunol. 165:5537.*
21. Liu, Z. X., S. Govindarajan, S. Okamoto, and G. Dennert. 2000. Fas- and tumor necrosis factor receptor 1-dependent but not perforin-dependent pathways cause injury in livers infected with an adenovirus construct in mice. *Hepatology 31:665.*
22. Brown, G. R., D. L. Thiele, M. Silva, and B. Beutler. 1997. Adenoviral vectors given intravenously to immunocompromised mice yield stable transduction of the colonic epithelium. *Gastroenterology 112:1586.*
23. Kolls, J. K., D. Lei, S. Nelson, W. R. Summer, S. Greenberg, and, B. Beutler.1995. Adenovirus-mediated blockade of tumor necrosis factor in mice protects against endotoxic shock yet impairs pulmonary host defense. *J. Infect. Dis. 171:570.*
24. Ullman, A., F. Jacob, and J. Monod. 1967. Characterization by in vitro complementation to an operator proximal segment of the β-galactosidase structural gene of *Escherichia coli. J. Mol. Biol. 24:339.*
25. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150:76.*
26. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol. 13:29.*
27. Ten Hagen, T. L., W. Van Vianen, and I. A. Bakker-Woundnberg. 1996. Isolation and characterization of murine Kupffer cells and splenic macrophages. *J. Immunol. Methods. 193:81.*
28. Ishii, M., B. Vroman, and N. F. LaRusso. 1989. Isolation and morphologic characterization of bile duct epithelial cells from normal rat liver. *Gastroenterology 97:1236.*
29. Lindroos, P. M., R. Zarnegar, and G. K. Michalopoulos. 1991. Hepatocyte growth factor (hepatopoietin A) rapidly increases in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbon tetrachloride administration. *Hepatology 13:743.*
30. Watanabe, H., K. Ohtsuka, M. Kimura, Y. Ikarashi, K. Ohmori, A. Kusumi, T. Ohteki, S. Seki, and T. Abo. 1992. Details of an isolation method for hepatic lymphocytes in mice. *J. Immunol. Methods 146:145.*
31. Goossens, P. L., H. Jouin, G. Marchal, and G. Milon. 1990. Isolation and flow cytometric analysis of the free lymphomyeloid cells present in murine liver. *J. Immunol. Methods 132:137.*
32. Miltenyi, S., W. Muller, W. Weichel, and A. Radbruch. 1990. High gradient magnetic cell separation with MACS. *Cytometry 11:231.*
33. Badovinac, V. P., and J. T. Harty. 2000. Intracellular staining for TNF and IFN-γ detects different frequencies of antigen specific $CD8^+$ T cells. *J. Immunol. Methods 238:107.*
34. McGuire, M. J., P. E. Lipsky, and D. L. Thiele. 1992. Purification and characterization of dipeptidyl peptidase I from human spleen. *Arch. Biochem. Biophys. 295:280.*
35. Green, G. D. J., and E. Shaw. 1979. Thiobenzyl benzyloxycarbonyl-1-lysinate, substrate for a sensitive colorimetric assay for trypsin-like enzymes. *Anal. Biochem. 93:223.*

36. Brown, G. R., K. Meek, Y. Nishioka, and D. L. Thiele. 1995. CD27-CD27 ligand/CD70 interactions enhance alloantigen-induced proliferation and cytolytic activity in $CD8^+$ T lymphocytes. *J. Immunol. 154:3686.*
37. Matzinger, P. 1991. The JAM test: a simple assay for DNA fragmentation and cell death. *J. Immunol. Methods 145:185.*
38. Wu, J. C., G. Merlino, and N. Fausto. 1994. Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor α. *Proc. Natl. Acad. Sci. USA 91:674.*
39. Brown, G. R., M. J. McGuire, and D. L. Thiele. 1993. Dipeptidyl peptidase I is enriched in granules of in vitro- and in vivo-activated cytotoxic T lymphocytes. *J. Immunol. 150:4733.*
40. Kondo, T., T. Suda, H. Fukuyama, M. Adachi, and S. Nagata. 1997. Essential roles of the Fas ligand in the development of hepatitis. *Nat. Med. 3:409.*
41. Galle, P. R., W. J. Hofmann, H. Walczak, H. Schaller, G. Otto, W. Stremmel, P. H. Krammer, and L. Runkel. 1995. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J. Exp. Med. 182:1223.*
42. Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature 364:806.*
43. Tanaka, M., T. Suda, T. Yatomi, N. Nakamura, and S. Nagata. 1997. Lethal effect of recombinant human Fas ligand in mice pretreated with *Propionibacterium acnes*. *J. Immunol. 158:2303.*
44. Froidevaux, S., L. Kuntz, D. Velin, and F. Loor. 1991. Different nature of the proliferation defects of *GLD*, LPR and MEV C57BL/6 mouse lymphoid cells. *Autoimmunity 10:233.*
45. Spriggs, M. K. 1996. One step ahead of the game: viral immunomodulatory molecules. *Annu. Rev. Immunol. 14:101.*
46. Lehmann, C., M. Zeis, N. Schmitz, and L. Uharek. 2000. Impaired binding of perforin on the surface of tumor cells is a cause of target cell resistance against cytotoxic effector cells. *Blood 96:594.*
47. Motyka, B., G. Korbutt, M. J. Pinkoski, J. A. Heibein, A. Caputo, M. Hobman, M. Barry, I. Shostak, T. Sawchuk, C. F. Holmes, et al. 2000. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell 103:491.*
48. Jans, D. A., V. R. Sutton, P. Jans, C. J. Froelich, and J. A. Trapani. 1999. BCL-2 blocks perforin-induced nuclear translocation of granzymes concomitant with protection against the nuclear events of apoptosis. *J. Biol. Chem. 274:3953.*
49. Bird, C. H., V. R. Sutton, J. Sun, C. E. Hirst, A. Novak, S. Kumar, J. A. Trapani, and P. I. Bird. 1998. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol. Cell. Biol. 18:6387.*