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Protection Against Polyoma Virus-induced Tumors is Perforin-independent

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Abstract

CD8 T cells are necessary for controlling tumors induced by mouse polyoma virus (PyV), but the effector mechanism(s) responsible have not been determined. We examined the PyV tumorigenicity in C57BL/6 mice mutated in Fas, or carrying targeted disruptions in the perforin gene or in both TNF receptor type I and type II genes. Surprisingly, none of these mice developed tumors. Perforin/Fas double-deficient radiation bone marrow chimeric mice were also resistant to PyV-induced tumors. Anti-PyV CD8 T cells in perforin-deficient mice were found not to differ from wild type mice with respect to phenotype, capacity to produce cytokines, or maintenance of memory T cells, indicating that perforin does not modulate the PyV-specific CD8 T cell response. In addition, virus was cleared and persisted to similar extents in wild type and perforin-deficient mice. In summary, perforin/granzyme exocytosis is not an essential effector pathway for protection against PyV infection or tumorigenesis.

Keywords

Polyoma virus; CD8 T cell; perforin; Fas; TNF; tumor; pathogenesis

Introduction

Polyoma virus (PyV) is a prevalent mouse pathogen that is capable of potent oncogenicity when inoculated into immunocompromised adult mice or newborn mice of certain strains (Law et al., 1967; Dawe et al., 1987). Early studies using thymectomized or congenitally athymic mice established that T cells mediate resistance against PyV-induced tumors (Law, 1965; Allison et al., 1974). Class I MHC-restricted virus-specific CD8 T cells have been implicated as anti-polyoma-tumor immune effectors (Greene et al., 1982; Ljunggren et al., 1994). In addition, susceptibility to PyV tumorigenesis results from a hole in the PyV-specific CD8 T cell repertoire created by an endogenous superantigen (Lukacher et al., 1995; Lukacher and Wilson, 1998). Finally, Drake et al. demonstrated that $\beta_2m^{\text{-/-}}$ mice are highly susceptible to PyV-induced tumors, providing the first direct evidence that class I MHC-restricted T cells prevent tumors resulting from PyV infection (Drake and Lukacher, 1998).
The long-term persistence of PyV DNA in multiple organs of adult-inoculated mice, together with evidence that priming of naïve virus-specific CD8 T cells occurs during persistent infection, indicates that PyV-specific CD8 T cells repetitively encounter viral antigen during the persistent phase of PyV infection (Drake and Lukacher, 1998; Kemball et al., 2005). Antiviral memory-stage CD8 T cells display extremely efficient in vivo cytotoxicity against peptide epitope-pulsed spleen cells (Barber et al., 2003; Byers et al., 2003). In the context of a persistent PyV infection, CD8 T cell-mediated cytotoxicity was considered essential for limiting viral replication and, in the case of non-productive viral replication, tumor formation. These studies implicated the perforin-granzyme exocytosis pathway as the dominant mechanism for clearance of antigen-pulsed spleen cells in vivo (Byers et al., 2003).

The dependence on perforin in host resistance to viral infection, however, varies with the pathogen (Mullbacher et al., 2004). Perforin-mediated cytotoxicity is essential for control of infection by LCMV, ectromelia virus, Ebola virus, or Theliers’s virus (; Kagi et al., 1994; Mullbacher et al., 1999; Gupta et al., 2005; Rossi et al., 1998; Walsh et al., 1994), while it is not necessary for controlling other viral infections (Franco et al., 1997; Kagi et al., 1995; Topham et al., 1997). Whether the viral life cycle is cytopathic (e.g., PyV) or noncytopathic (e.g., hepatitis B virus, LCMV) does not predict a requirement for perforin in antiviral immunity (Mullbacher et al., 2004), and the extent of viral control by perforin-deficient mice can vary even between infections by closely related viral infections (Mullbacher, 2003).

Immune mechanisms that control viral replication during persistent viral infections may also vary between acute and persistent phases of infection. For example, during murine cytomegalovirus (MCMV) infection, viral replication in the salivary glands is dependent on perforin during acute infection while control of persistent MCMV infection is independent of perforin-mediated exocytosis (Riera et al., 2000). Alternatively, control of γHV68 infection is not affected by loss of perforin during the acute immune response (Topham et al., 2001; Usherwood et al., 1997), but perforin-deficient mice have increased numbers of latently infected cells compared to wild type mice (Tibbetts et al., 2002).

In this study, we investigated the contribution of perforin/granzyme exocytosis to the control of viral replication during acute and persistent phases of PyV infection. In particular, we sought to determine whether perforin-mediated cytotoxicity represented the dominant pathway engaged by CD8 T cells to prevent PyV tumorigenesis. Unexpectedly, we found that perforin-deficient mice efficiently limited acute PyV infection and retained resistance to PyV-induced tumors. In addition, PyV-specific CD8 T cell responses in these mice were indistinguishable from those of wild type mice. Thus, we found no evidence for an immunomodulatory role for perforin in the CD8 T cell response to PyV infection or for perforin/granzyme exocytosis as an essential antiviral or antitumor effector mechanism against this mouse pathogen.

Results

Analyses of PyV tumor induction in perforin, Fas, and TNF receptor-deficient mice

To identify candidate cytotoxic effector mechanisms that could confer protection from PyV-induced tumors, adult C57BL/6 mice mutated in Fas (lpr), or carrying targeted disruptions in the perforin gene (PKO) or in both TNF receptor type I and type II genes (TNFRKO) were inoculated subcutaneously with PyV and monitored for tumors. MHC class I-deficient C57BL/6.β2m−/− mice and wild type C57BL/6 mice served as controls for PyV-induced tumor susceptibility and resistance, respectively. Seven of ten C57BL/6.β2m−/− mice developed tumors within 3 months of PyV infection (Table 1). Tumors in these mice almost
exclusively arose in salivary and mammary glands, which are among the most frequent types of PyV-induced tumors, particularly in immunocompromised adult mice (Berebbi et al., 1988; Drake and Lukacher 1998). During the same 3-month period, the 3 overtly tumor-free C57BL/6.β2−/− mice developed hind-limb paralysis, presumably caused by a vertebral bone tumor (Wirth et al., 1992). None of the Fas-defective, TNFRKO, or PKO mice developed tumors during the 6-month monitoring period. Because high susceptibility to PyV-induced tumors is realized only in particular inbred strains of immunocompetent mice when inoculated at birth, we also monitored the incidence of PyV-induced tumor growth in neonatally-infected PKO and wild type C57BL/6 mice. Neither newborn-infected PKO nor wild type mice developed tumors (Table 1). Taken together, these data indicate that the FasL-Fas, perforin/granzyme, and TNFR-mediated effector pathways are individually dispensable in controlling PyV tumorigenesis.

Because PKO mice retained resistance to PyV-induced tumors, we next asked whether PKO and wild type mice differed in efficiency of viral clearance. Viral plaque assays performed on splenic homogenates showed similar peak titers and rates of clearance of infectious virus by PKO and wild type mice during acute infection (Fig. 1A). Real-time PCR for PyV DNA showed that there were no significant differences between wild type and PKO mice acute viral load in spleen, lung, or liver (Fig. 1B). Interestingly, compared to persistently infected wild type mice, persistently infected PKO mice had comparable viral loads in the spleen but harbored significantly higher amounts of viral DNA in lung and heart (Fig. 1C). We recently reported that in vivo blockade of CD28 and CD40 ligand costimulatory pathways is similarly associated with interorgan variability in PyV load (Kemball et al., 2006); however, the mechanisms regulating organ-specific immune control of persistent PyV infection remain to be defined.

**PyV-specific CD8 T cell response in PKO mice**

PKO mice infected with either LCMV or *Listeria* display massive expansion of activated Ag-specific CD8 T cells (Kagi et al., 1999; Matloubian et al., 1999; Badovinac et al., 2000), implicating a downmodulatory role for perforin in CD8 T cell responses during acute infection. In PyV-infected PKO mice, however, total numbers of splenic CD8 T cells specific for the dominant Dβ-restricted LT359 epitope were similar to that seen in wild type C57BL/6 mice during the peak of the acute response, the contraction phase, and the persistent-infection phase (Fig. 2A). Additionally, total numbers of PyV-specific CD8 T cells in the liver and lungs of PKO and wild type mice were not substantially different (data not shown). No overt phenotypic differences distinguished PyV-specific CD8 T cells in PKO and wild type mice. Nearly identical frequencies of LT359-specific CD8 T cells in PKO and wild type mice expressed the IL-7Ra (CD127), the IL-2/IL-15β receptor (CD122), and the TNF receptor family member CD27 during the course of PyV infection (Fig. 2B). PKO and wild type mice had a similar anti-PyV CD8 T cell hierarchy for the dominant LT359 and subdominant MT246 and LT638 epitopes (Kemball et al., 2005) (data not shown).

Production of granzyme B, a serine protease that resides in cytotoxic granules with perforin, also did not differ between wild type and PKO mice during the acute PyV immune response (Fig. 3A). Additionally, total numbers of LT359 peptide-stimulated CD8 T cells expressing surface CD107a, a lysosomal membrane protein expressed on the surface of degranulating T cells (Betts et al., 2003), were similar throughout the immune response in both mice (data not shown). LT359-peptide stimulation elicited similar frequencies of CD8 T cells producing the antiviral cytokines IFN-γ and TNF-α in both wild type and PKO mice (Fig. 3B). These phenotypic and functional data suggest that PyV-specific CD8 T cells in PKO mice complete the same program of differentiation as those in infected wild type mice.
In vivo antigen-specific cytotoxicity in PKO mice

Lack of tumor formation in mice deficient in components of the Fas/FasL and TNF/TNFR pathways (Table 1) could indicate that absence of an individual CTL effector pathway may not be sufficient to render mice susceptible to PyV-induced tumors. In support of the potential requirement for multiple cytotoxic effector modalities in antiviral clearance, Topham and colleagues showed that deficiency in both perforin and Fas was required to negate control of pulmonary γHV68 replication (Topham et al., 2001). Although acutely PyV-infected Fas-deficient mice killed LT359 peptide-pulsed targets in vivo as efficiently as wild type mice, acutely infected PKO mice retained substantial in vivo LT359-specific cytotoxicity, albeit at much lower levels than in C57BL/6 mice. This finding raised the possibility for redundancy in these two CTL effector pathways (Byers et al., 2003). To test this, we found that LT359 peptide-pulsed lpr splenic target cells were eliminated with markedly reduced efficiency compared to identically treated wild type targets in PKO mice. This result supports the likelihood that PyV-specific CD8 T cells engage both perforin/granzyme exocytosis and Fas/FasL effector pathways in infected mice (Fig. 4A).

In wild type mice, antiviral memory CD8 T cells rapidly mobilize potent in vivo cytotoxic function to nearly the same extent as antiviral CD8 T cells from acutely infected mice (Byers et al., 2003). Acutely infected PKO mice eliminate approximately 30% of LT359 peptide-pulsed targets during a 4-hour in vivo CTL assay (Byers et al., 2003). However, over the same time period, virtually none of the peptide-pulsed targets are eliminated in persistently infected PKO mice (Fig. 4B). This data indicates that alternative effector pathways do not compensate for the absence of perforin during the persistent phase of PyV infection.

Analysis of PyV tumorigenesis in absence of both Fas and perforin

To more definitively test the in vivo effects of eliminating both perforin- and Fas/FasL-mediated effector pathways in PyV-infected mice, we created radiation bone marrow chimeras doubly deficient in Fas and perforin, as previously described (Topham et al., 2001). Lethally irradiated wild type C57BL/6 or Fas-deficient B6.lpr mice were reconstituted with bone marrow cells from PKO mice, infected with PyV and monitored for virus levels and tumor formation. Reconstitution of wild type mice with PKO bone marrow cells served as a control to determine whether perforin-deficient radiation bone marrow chimeras recapitulate the effective antiviral immune response seen in gene targeted PKO mice. PCR analysis confirmed that these reconstituted mice contained the same mutated pfp gene as the PKO mice (data not shown). The use of this radiation bone marrow chimera approach circumvents the spontaneous autoimmunity and early mortality seen in perforin/FasL double-deficient mice (Spielman et al., 1998). Six months after PyV infection, none of the PKO:lpr chimeric mice developed overt tumors. PKO:C57BL/6 chimeric mice could not be monitored for long-term tumor growth because all of these chimeric mice died between 8-16 weeks after PyV infection. None of these mice showed signs of overt tumor growth and no gross tumors were seen on necropsic examinations of several agonal mice (data not shown). In addition, these mice showed marked morbidity (i.e., runting, hunched posture, and dyspnea), symptoms not seen in unirradiated PKO mice. Three of 9 PyV-infected PKO:lpr chimeras also showed signs of morbidity between 20-32 weeks postinfection and necropsies of these mice showed no overt tumors (data not shown). The absence of PyV-induced tumors in the remaining PKO:lpr chimeras indicates that non-perforin/Fas-mediated effector mechanisms are responsible for an immunosurveillance for PyV-induced tumors. It is interesting to note that Topham et al. described similar deleterious outcomes in chimeric perforin-deficient, Fas-sufficient mice inoculated intranasally with γHV68, and raised the possibility that irradiation may increase expression levels of Fas in the lung and consequently increased levels of Fas/FasL-mediated lysis (Topham et al., 2001). Because
PyV remained a silent infection in unirradiated PKO mice, the higher incidence of lethality in the PKO:C57BL/6 than the PKO:lpr chimeric mice is consistent with this suggestion that Fas contributes to this late-appearing morbidity/mortality.

**Discussion**

The controversial concept that immunosurveillance protects against spontaneously developing tumors has garnered strong support from recent studies in humans and in mouse models (Dunn et al., 2004). Perforin-mediated cytotoxicity is the hallmark CD8 T cell effector mechanism in vitro (Kagi et al., 1994; Walsh et al., 1994) and represents the dominant pathway for CTL lysis in vivo (Barber et al., 2003; Byers et al., 2003). Moreover, there is strong evidence documenting the importance of perforin-mediated cytotoxicity in tumor rejection (van den Broek et al., 1996; Smyth et al., 1999; van Elsas et al., 2001) and control of spontaneous malignancies (Smyth et al., 2000). Thus, the central finding here, that perforin-deficient mice efficiently cleared infectious virus and were highly resistant to PyV-induced tumors, argues against an independent role for perforin/granzyme exocytosis in PyV pathogenesis.

Perforin-deficient PyV-specific CD8 T cells appear to have fully developed secretory lysosomal compartments, because ex vivo intracellular granzyme B staining and cognate peptide-induced degranulation in PKO mice are comparable to wild type C57BL/6 mice (Fig. 2C and data not shown). Although in vivo cytotoxicity is largely perforin-dependent, a substantial amount of antigen-specific killing was seen in PKO mice, suggesting that a compensatory cytotoxicity pathway(s) enables CD8 T cells to eliminate virally infected cells in the absence of perforin. Decreased in vivo killing in the absence of both perforin and Fas indicates that both of these effector pathways are operative in the anti-PyV immune response (Fig. 4A). Because PKO:lpr bone marrow chimeric mice are resistant to PyV-induced tumors, however, CTL effector functions other than perforin- and Fas/FasL-mediated cytolysis must confer tumor immunosurveillance. IFN-γ represents a candidate anti-tumor CD8 T cell effector mechanism. Rejection of transplanted tumors has been shown to be markedly less effective in IFN-γ-deficient recipient mice in situations where deficiencies in any of the three major cytotoxic effector pathways (i.e., perforin-, Fas ligand- and TNF-α-mediated cytotoxicity) have little effect (Dobrzanski et al., 2004; Hollenbaugh et al., 2004). Alternatively, IFN-γ may be required in concert with CD8 T cell cytotoxicity effector activity to limit persistent PyV infection and confer protection from PyV-induced tumors. By secreting cytokines and chemokines, antigen-specific CD8 T cells may also indirectly mediate antiviral and antitumor surveillance by recruiting and activating other effector cells (e.g., macrophages, neutrophils).

The absence of in vivo antigen-specific cytotoxicity in persistently infected PKO mice (Fig. 3B) suggests that Fas/FasL-mediated cytotoxicity may predominantly operate during the acute infection phase of the anti-PyV immune response. In contrast to the TCR-dependent engagement of perforin/granzyme exocytosis, cytokines are capable of upregulating surface expression of both Fas on target cells and FasL on CD8 T cells (Esser et al., 1997). The inflammatory cytokine-rich milieu during acute infection may thereby superimpose FasL/Fas cytotoxicity on the perforin-mediated pathway to most efficiently clear large-scale infection. In contrast, lack of bystander Fas/FasL-mediated cytotoxicity during the persistent phase of infection fits with the concept that virus-specific CD8 T cells must balance control of persistent viral replication against immunopathologic tissue damage.

We were unable to identify an immunomodulatory role for perforin in the anti-PyV CD8 T cell response. The exaggerated expansion of antigen-specific CD8 T cells during acute LCMV or *Listeria monocytogenes* infection suggested that perforin downregulates CD8 T
cell responses in concert with viral clearance (Kagi et al., 1999; Matloubian et al., 1999).
Subsequent studies, however, showed that absence of perforin-mediated lysis of epitope-
expressing dendritic cells was associated with progressive expansion in antigen-specific
CD8 T cell numbers (Badovinac et al., 2000; Badovinac et al., 2003; Yang et al., 2006),
raising the question as to whether persistent antigen drives uncontrolled CD8 T cell
expansion in PKO mice. Similar to our findings, Christensen et al. recently reported that
PKO mice efficiently clear vesicular stomatitis virus infection and mount virus-specific CD8
T cell responses comparable to wild type mice (Christensen et al., 2004). Thus, whether
perforin modulates CD8 T cell responses appears to depend on the particular host-pathogen
interaction being studied.

In summary, the findings described here demonstrate that perforin is dispensable for limiting
PyV infection or conferring resistance to PyV-induced tumors. This study indicates that
other effector mechanisms, perhaps together with perforin/granzyme exocytosis, are
required to mediate surveillance for infected/transformed cells in PyV-infected hosts.

Materials and Methods

Mice

C3H/HeNCr and C57BL/6NCr mice were purchased from the National Cancer Institute.
B6.MRL-Tnfrsf6flp, B6.129S-Tnfrsf1atm1ImxTnfrsf1btm1Imx, C57BL/6-Pfpmt1Sdz, and
C57BL/6.β2m−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME).
Mice were housed and bred in accordance with the guidelines of the Institutional Animal
Care and Use Committee, and the Department of Animal Resources at Emory University.

Virus and inoculations

PyV strain A2 was molecularly cloned, plaque purified, and virus stocks were prepared on
baby mouse kidney cells, as previously described (Lukacher and Wilson, 1998). Adult mice
(6-12 weeks of age) were inoculated subcutaneously in each hind footpad with 1 × 10⁶ PFU
of virus. Newborn mice (<12 hours of birth) were inoculated subcutaneously in each hind
footpad with 2 × 10⁵ PFU of virus.

In vivo CTL assay

This assay was performed as previously described (Byers et al., 2003). Briefly, peptide-
pulsed spleen cells were co-stained with PKH26 and differential concentrations of CFSE
and equal numbers of peptide-pulsed and unpulsed cells were adoptively transferred into
infected or naïve syngeneic recipients. At 4 hours after transfer, mice were sacrificed, organs
harvested and single cell suspensions of spleen were analyzed by flow cytometry. Percent
specific lysis of fluorescent donor spleen cells in each mouse is calculated as follows:
((number of unpulsed targets × A – number of peptide-pulsed targets)/ number of unpulsed
targets × A) × 100, where A= (number of unpulsed targets/number of peptide-pulsed targets)
in uninfected recipient mice.

Plaque assay

Spleen samples were snap frozen in sterile Kontes tubes (Kontes, Vineland, NJ), adjusted to
50 mg/ml in DMEM at 4°C, and homogenized using an overhead stirrer (Wheaton,
Millville, NJ) and disposable Teflon pestles (Kontes). Homogenized tissues were then
freeze-thawed three times, incubated for 45 minutes at 42°C, and centrifuged to remove cell
debris. Supernatants were titered for infectious virus by plaque assay on BALB/3T3 clone
A31 cells. The detection limit for this plaque assay is 1 PFU/mg spleen.
Taqman real-time PCR

DNA was extracted from whole blood or snap-frozen tissue using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. Taqman PCR was performed as described (Kemball et al., 2005). The PyV DNA quantity is expressed in genome copies per milligram of tissue and is calculated based on a standard curve of known PyV genome copy number vs. threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

Flow cytometry

RBC-lysed spleen cells were stained with allophycocyanin-conjugated Dβ/LT359-LT638 (with the cysteine at position 7 replaced by α-aminobutyric acid, a thiol group-less cysteine analog) tetramers (prepared as described in (Kemball et al., 2006)) and the following mAbs: FITC-conjugated anti-CD8α (BD Biosciences) and PE-conjugated mAbs to CD27, CD122, and CD127 (eBioscience). For intracellular staining, spleen cells were cultured for 4 hours in 96-well round-bottom microtiter plates in IMDM (Invitrogen) containing 10% FBS, penicillin/streptomycin, 50 μM 2-ME, synthetic peptides, and 1 μg/ml brefeldin A (BFA). Cells were then stained for surface markers, washed, permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained intracellularly using allophycocyanin-conjugated anti-IFN-γ (XMG 1.2; BD Biosciences) or PE-conjugated anti-TNFα (MP6-XT22; Caltag). Staining for Granzyme B (GB12; Caltag) or its isotype control (mouse IgG1) was carried out directly ex vivo on cells permeabilized as above. Degranulation assays measuring surface expression of CD107a and CD107b were carried out as described (Betts et al., 2003). FITC-conjugated CD107a and CD107b were purchased from BD Biosciences. Samples were acquired on a FACSCalibur (BD Biosciences) and data were analyzed using CellQuest (BD Biosciences) and FlowJo (TreeStar) software.

Generation of radiation bone marrow chimeras

B6.MRL-Tnfrsf6f6pr mice or wild type C57BL/6 mice were lethally irradiated with 1000 rad and reconstituted 12 h later with 2 × 10⁷ bone marrow cells taken from the femurs of C57BL/6-Pfpim1Sdz mice. Mice were rested for 10 weeks to allow for hematopoietic cell reconstitution before s.c. infection in hind footpads with 1 × 10⁶ PFU of PyV. Complete engraftment by PKO bone marrow was confirmed by PCR using oligonucleotide primers and annealing conditions as described (Bergmann et al., 2003). Assessment of PyV DNA levels in blood and tissues of mice was performed at necropsy and mice were monitored for signs of tumor growth.

Statistics

Statistical significance was determined by an unpaired Student’s t test, assuming unequal variances. A p value of <0.05 was considered statistically significant.

Acknowledgments

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References


Fig. 1.
PyV clearance in wild type and PKO mice. (A) Spleens from wild type (C57BL/6) and PKO mice at the indicated days postinfection were harvested, homogenized, and titered for PyV by plaque assay. Each value represents the mean ± SEM PFU/mg from 3 individual mice and is representative of 2 experiments. *, below detection limit of 1 PFU/mg. (B) and (C) Levels of PyV DNA in the indicated organs of C57BL/6 or PKO mice during acute (B, day 8 postinfection) or persistent (C, > 40 days postinfection) phase of infection were quantitated by Taqman real-time PCR. Student’s t-test values between C57BL/6 and PKO: acute spleen $p = 0.29$, acute lung $p = 0.30$, acute heart $p = 0.62$; persistent spleen $p = 0.31$,
persistent lung $p < 0.001$, persistent heart $p = 0.03$. Mean ± SEM values for 3 mice per group are shown and data are representative of 2 experiments.
Fig. 2.
Visualization of the PyV-specific CD8 T cell response in PKO mice. (A) Spleen cells from PKO (■) or C57BL/6 (◆) mice at the indicated day after infection were stained ex vivo with anti-CD8α and Db LT359 tetramers and analyzed by flow cytometry. (B) Spleen cells from PKO (■) or C57BL/6 (◆) mice at the indicated day after infection were stained ex vivo with anti-CD8α, Db LT359 tetramers, and anti-CD127, anti-CD122, or anti-CD27 and analyzed by flow cytometry. Data are representative of 3 experiments with 3 mice per experiment.
Fig. 3.
PyV-specific CD8 T cells in PKO and wild type mice are functionally similar. (A) Spleen cells from C57BL/6 or PKO mice at day 8 postinfection were stained ex vivo with anti-CD8α, D^b LT359 tetramers, and intracellularly for anti-granzyme B and analyzed by flow cytometry. Plots are gated on CD8 T cells and numbers indicate the percentage of cells in the indicated quadrant. Data are representative of 3 experiments with 3 mice per experiment. (B) Spleen cells from C57BL/6 and PKO mice at day 8 postinfection were stimulated with or without 10 μM LT359 peptide for 5 hours in the presence of BFA, then surface-stained with anti-CD8α and intracellularly stained for IFN-γ or TNF-α, and analyzed by flow cytometry.

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cytometry. Plots are gated on live lymphocytes. Axes of the plots are log of fluorescent intensity. Data are representative of 2-3 experiments with 3 mice per experiment.
Fig. 4. Dependence of PyV-specific in vivo CTL activity on perforin and Fas. (A) LT359 peptide-pulsed and unpulsed naïve C57BL/6, lpr, or TNFR−/− spleen cells were injected into C57BL/6 mice 7 days after PyV infection. Peptide-pulsed and unpulsed naïve spleen cells were injected into PKO mice 7 days postinfection. Peptide-pulsed and unpulsed lpr spleen cells were injected into PKO mice 7 days postinfection. (B) LT359 peptide-pulsed and unpulsed naïve C57BL/6 spleen cells were injected into C57BL/6 or PKO mice during the acute or persistent phase of the immune response. For both (A) and (B), values represent mean ± SEM percent specific lysis of peptide-pulsed spleen cells at 4 hours of 3 recipient mice in 2 separate experiments.
## Table 1

PyV tumorigenesis in mice deficient in perforin, Fas, or TNF receptors

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Age of Inoculation</th>
<th>Tumor Incidence$^a$</th>
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<tbody>
<tr>
<td>B6.β2m$^{-/-}$</td>
<td>adult</td>
<td>7/10</td>
</tr>
<tr>
<td>B6.PKO</td>
<td>adult</td>
<td>0/10</td>
</tr>
<tr>
<td>B6.lpr</td>
<td>adult</td>
<td>0/12</td>
</tr>
<tr>
<td>B6.TNFR$^{-/-}$</td>
<td>adult</td>
<td>0/12</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>adult</td>
<td>0/12</td>
</tr>
<tr>
<td>B6.PKO</td>
<td>newborn</td>
<td>0/11</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>newborn</td>
<td>0/10</td>
</tr>
</tbody>
</table>

$^a$ Adult-inoculated mice were monitored for 6 months; if free of palpable tumors, mice were necropsied for evidence of overt visceral tumors. Tumor-bearing B6.β2m$^{-/-}$ mice were necropsied at 9-13 weeks, when tumors (predominantly of the salivary gland) attained a size necessitating euthanasia. Newborn mice were inoculated by PyV within 24 hours of birth and were monitored for 4 months for development of tumors, then necropsied.