Immunity *24*, 451–461, April 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.02.002

Serine Protease Inhibitor 6 Protects Cytotoxic T Cells from Self-Inflicted Injury by Ensuring the Integrity of Cytotoxic Granules

Manling Zhang,[1,2] Sun-Mi Park,[2] Yue Wang,[1,2] Ramila Shah,[1,2] Ni Liu,[1,2] Andrea E. Murmann,[3] Chyung-Ru Wang,[1] Marcus E. Peter,[2] and Philip G. Ashton-Rickardt[1,2,*]
[1] Department of Pathology
[2] Ben May Institute for Cancer Research
[3] Department of Medicine
The University of Chicago
924 East 57th Street
Chicago, Illinois 60637

Summary

How cytotoxic T lymphocytes (CTLs) kill intracellular pathogens without killing themselves has been a recurring question ever since their discovery. By using mice deficient in Serine Protease Inhibitor 6 (Spi6), we show that by inhibiting granzyme B (GrB), Spi6 protects CTLs from self-inflicted injury. Infection with either Lymphocytic Choriomeningitis virus (LCMV) or *Listeria monocytogenes* (LM) revealed increased apoptosis and diminished survival of Spi6 knockout (KO) CTLs, which was cell autonomous and could be corrected by GrB deficiency. Spi6 KO mice in turn were impaired in their ability to clear LCMV infection. Spi6 KO CTLs revealed a breakdown in the integrity of cytotoxic granules, increased cytoplasmic GrB, and ensuing apoptosis. We conclude that Spi6 protects CTLs from suicide caused by GrB-mediated breakdown of cytotoxic granules.

Introduction

Contact-dependent, lymphocyte-mediated cytotoxicity proceeds through two pathways. The first pathway is triggered by members of the Tumor Necrosis Factor Receptor family, of which Fas is the most important (Kagi et al., 1996). The second involves the exocytosis of proteins present in $CD8^+$ cytotoxic T lymphocyte (CTL) and natural killer (NK) cell granules (Millard et al., 1984; Podack et al., 1985). The exocytosis pathway affords the rapid and targeted destruction of infected or damaged cells. Exocytosis of perforin (Pfn) (Kagi et al., 1994a; Podack et al., 1985) facilitates the entry of serine proteases called granzymes, which trigger apoptosis in target cells (Shi et al., 1992). Granzymes A and B are the most abundant granzymes in mice and humans and are the best characterized (Russell and Ley, 2002). Granzyme B (GrB) activates the caspase-dependent pathways of apoptosis and, like caspases, cleaves after aspartic acid residues, whereas granzyme A (GrA), which is a tryptase, cleaves after basic amino acids. Recent studies indicate that the mechanisms by which different granzymes operate are discrete, providing failsafe mechanisms to destroy tumors or pathogens that have devised ways to evade a given pathway (Lieberman, 2003).

*Correspondence: pashtonr@midway.uchicago.edu

Given the effectiveness of the granule exocytosis pathway of death, it is not surprising that CTLs and NK cells are susceptible to self-inflicted damage. The role of the exocytosis pathway in CTL apoptosis has been clearly shown by Pfn-deficient CTLs, which are defective in granule-mediated killing (Kagi et al., 1994a, 1994b) but also undergo less apoptosis in vitro (Spaner et al., 1998). When challenged in vivo with allografts (Spaner et al., 1999), viruses, or intracellular bacteria (Badovinac et al., 2000, 2003; Kagi et al., 1999; Matloubian et al., 1999), Pfn-deficient CTLs undergo a massively increased expansion compared to wild-type controls.

It is unclear how granzymes are turned against CTLs. The traffic of granzymes into a target cell is achieved by the unidirectional exocytosis of cytotoxic granules into the secretory synapse (Kupfer et al., 1986). Therefore, the simplest explanation for CTL self-destruction is that CTLs become the targets for killing by other CTLs (Hanon et al., 2000; Huang et al., 1999; Walden and Eisen, 1990) (fratricide). Although the delivery of granzymes to target cells is highly ordered (Trambas and Griffiths, 2003), endogenous granzymes may become "misdirected" into the cytoplasm and so a cytotoxic lymphocyte may die through suicide (Ida et al., 2003). Despite the propensity for self-inflicted damage, CTLs can kill several targets in succession without killing themselves (Zagury et al., 1975). Therefore, it has been suggested that cytotoxic lymphocytes can protect themselves from self-inflicted damage (Trambas and Griffiths, 2003).

Homeostatic regulation of serine proteases is mainly achieved through interaction with inhibitors belonging to the Serine Protease Inhibitor (serpin) superfamily (Silverman et al., 2001). Inhibitory serpins have a common mode of action: each contains a variable C-terminal reactive center loop (RCL) resembling the substrate of its cognate protease. On protease binding, the RCL is cleaved between the two residues designated P_1 and P'_1 and it undergoes a conformational change that distorts the protease and irreversibly locks the serpin-protease complex. The human serpin, Proteinase Inhibitor 9 (PI9), is a potent inhibitor of GrB (Sun et al., 1996). In mice, Serine Protease Inhibitor 6 (Spi6), a homolog of PI9, can also inhibit GrB in vitro (Sun et al., 1997) and is expressed in CTLs and NK cells (Phillips et al., 2004). Both PI9 and Spi6 lack signal secretory sequences and so are located in the cytoplasm (Sun et al., 1996, 1997).

Spi6 is upregulated upon the maturation of dendritic cells (DCs) (Medema et al., 2001a), and overexpression protects targets from CTL killing (Medema et al., 2001b). Transgenic Spi6 can increase the number of CD8 T cells that persist long after virus clearance (Phillips et al., 2004). However, GrB KO mice do not show any increase in the number of CTLs after infection with virus and so it is not clear if GrB induces CTL apoptosis (Phillips et al., 2004). Therefore, whether inhibition of GrB is a physiologically relevant mechanism by which Spi6 protects CTLs remains to be determined. To test this hypothesis, we generated Spi6-deficient mice (Spi6 knockout [KO] mice). The survival of Spi6 KO

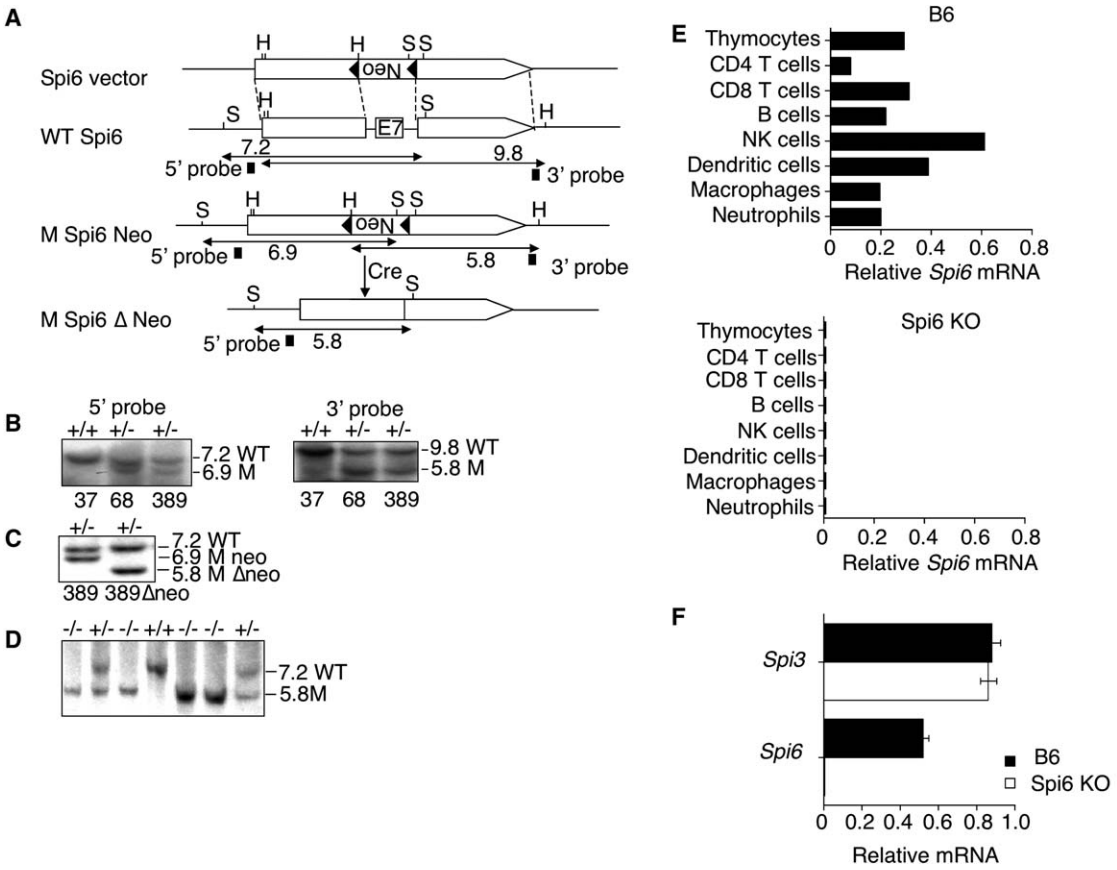

Figure 1. Spi6-Deficient Mice

(A) Homologous recombination between the *Spi6* targeting vector and wild-type allele in ES cells as detected by altered restriction fragments [*Hind III* (H), *Spe I* (S)] using 5′ and 3′ external probes. *Cre*-induced *loxP* (filled arrows) mediated excision of *neo*, resulting in restriction fragments of altered size (kb).

(B) Southern blots of two ES cell clones with wild-type (WT: 37) or mutant (M: 69 and 389) *Spi6 neo* alleles.

(C) A clone derived from 389 harboring an allele in which *neo* has been excised (*Spi6Δneo*).

(D) *Spi6*$^{+/-}$ mice were intercrossed and progeny genotyped by Southern blot.

(E) *Spi6* mRNA expression determined by real-time PCR and expressed as a ratio compared with the control *cyclofilin A*. Cells were purified from four mice and pooled and mRNA was prepared. Histograms are the mean of three determinations.

(F) *Spi3* and *Spi6* mRNA levels determined from splenocytes by real-time PCR as in (E). Histograms are the mean of two determinations from four mice (±SEM). There was no significant difference between the levels of *Spi3* mRNA in splenocytes from B6 compared to Spi6 KO mice (p = 0.85).

CTLs specific for LCMV or LM was drastically impaired due to increased GrB-mediated apoptosis. Spi6 protected CTLs from apoptosis by inactivating GrB in the cytoplasm through the formation of SDS-stable complexes indicative of a serpin:protease interaction. To our surprise, Spi6 was required to ensure the integrity of exocytic cytotoxic granules through the inhibition of GrB-mediated breakdown. These findings demonstrate the requirement for an endogenous inhibitor of GrB to protect CTLs from suicide caused by granule breakdown.

Results

Impaired Survival of Spi6-Deficient CTLs

To address the role of the GrB inhibitor Spi6 (*Serpin b9*) (Sun et al., 1997) in the protection of CTLs from self-inflicted damage, we generated *Spi6*-deficient mice. By means of homologous recombination in ES cells from C57BL/6 mice (B6 mice), we deleted exon 7, which encodes 60% of *Spi6* and includes the critical reactive center loop (RCL), which is required for target protease inhibition (Sun et al., 1997) (Figures 1A and 1B). By Cre-mediated recombination, the G418-resistance cassette was removed (Figure 1C) to avoid affecting the transcription of closely linked serpin genes such as *Spi3 (Serpin b6a)* (Sun et al., 1997; Kaiserman et al., 2002). Mice homozygous for *Spi6* mutant alleles in the B6 background (Spi6 KO mice) were generated (Figure 1D). Real-time PCR for the *Spi6* RCL region revealed the expression of *Spi6* mRNA in both myeloid and lymphoid cells from B6 but not Spi6 KO mice (Figure 1E). The mutation of the *Spi6* locus did not affect the expression of *Spi3* mRNA in Spi6 KO splenocytes (Figure 1F). The development of leukocytes was not affected by the *Spi6* mutation because Spi6 KO mice did not display any significant difference in the number of myeloid or lymphoid cells in the thymus or blood (see Tables S1 and S2 in the Supplemental Data available with this article online).

Spi6 is upregulated in CTLs, and overexpression can inhibit GrB-mediated apoptosis in vitro (Phillips et al., 2004). To examine the physiological role of Spi6, we examined the CTL response of Spi6 KO mice to infection.

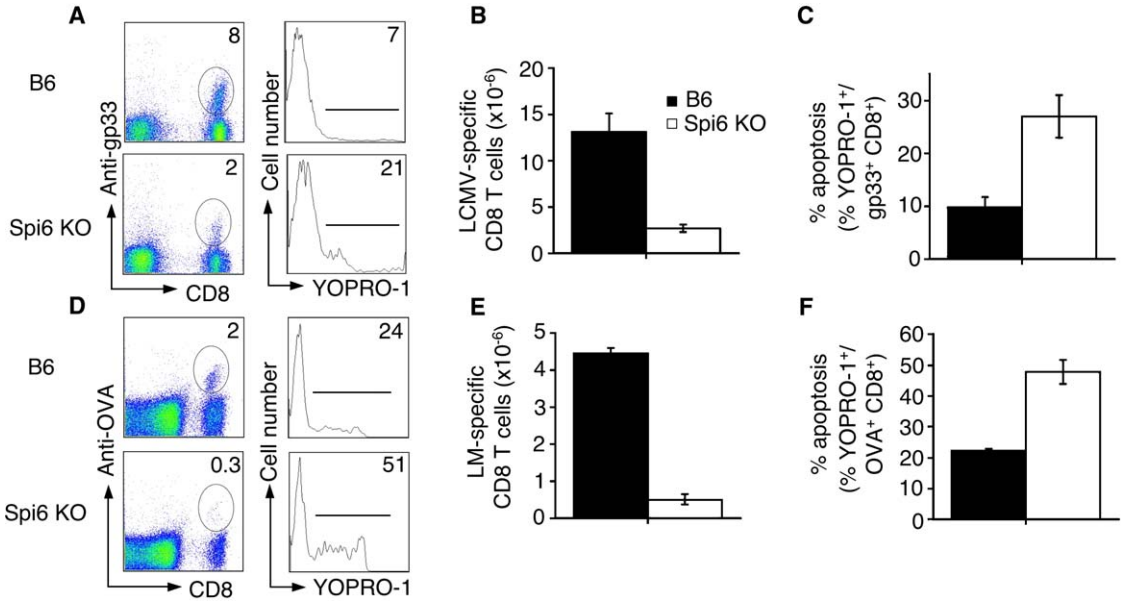

Figure 2. Impaired Survival and Function of Spi6 KO CTLs

B6 or Spi6 KO mice were infected with either LCMV Armstrong (2×10^5 pfu/mouse i.p.) or LM DPL-1942-OVA (10^5 cfu/mouse i.v.).
(A) Percentage (upper right corner) of gp33$^+$ CD8$^+$ and YOPRO-1$^+$ cells.
(B) Mean number gp33$^+$ CD8$^+$ cells ± SEM (n = 5 mice).
(C) Mean percentage YOPRO-1$^+$ of gp33$^+$ CD8$^+$ ± SEM (n = 5 mice).
(D) Percentage OVA$^+$ CD8$^+$ and YOPRO-1$^+$ cells.
(E) Mean number of OVA$^+$ CD8$^+$ spleen cells ± SEM (n = 5 mice).
(F) Mean percentage of YOPRO-1$^+$ of OVA$^+$ CD8$^+$ ± SEM (n = 5 mice).

Mice were infected with LCMV Armstrong (Phillips et al., 2004) and the attenuated DPL-1942 strain of LM, which had been engineered to express ovalbumin and generate an H-2K^b-restricted peptide antigen (OVA) (Pope et al., 2001). To avoid the effects of persistent antigen on interferon-driven apoptosis (Badovinac et al., 2000), we used doses of LCMV Armstrong (2×10^5 pfu) and LM DPL-1942 (10^5 cfu) that result in complete clearance in both B6 and Spi6 KO mice after 8 days. Staining with gp 33/H-2D^b-tetramers revealed that the number of CTL specific for LCMV Armstrong was diminished in Spi6 KO mice compared to B6 controls (5-fold lower, $p = 3 \times 10^{-9}$) (Figures 2A and 2B). Furthermore, Spi6 KO mice harbored about nine times ($p = 2 \times 10^{-11}$) less LM-specific CTLs after infection (Figures 2D and 2E). In contrast to the deficit in CTLs, there was no significant difference in the number of CD4 T lymphocytes, B lymphocytes, NK cells, or macrophages either 8 or 14 days after LCMV infection (Table S3).

The lower number of CTLs in Spi6 KO mice correlated with an increase in the onset of apoptosis of both LCMV (Figures 2A and 2C) and LM-specific CD8 T cells (Figures 2D and 2F), as evidenced by increased staining with the DNA dye YOPRO-1, which is a robust measure of apoptosis based on alterations in chromosomal DNA structure (Idziorek et al., 1995; Liu et al., 2004; Opferman et al., 2001; Phillips et al., 2004). Thus, Spi6 is required for the survival and protection of CTLs from apoptosis in vivo.

Defective Survival of Spi6 KO CTLs Is GrB Dependent and Cell Autonomous

We determined whether inhibition of GrB was a physiological mechanism by which Spi6 ensured the survival of CTLs. To do this, we examined the effect of GrB deficiency in Spi6 KO x GrB cluster-deficient (GrB KO) mice (Heusel et al., 1994). After infection, the number of LCMV-specific CTLs in GrB KO mice is the same as in wild-type mice (Phillips et al., 2004; Zajac et al., 2003), possibly because other homeostatic factors also act to maintain a normal clonal burst size (Jameson, 2002). As we observed before, there was a 4-fold decrease in the recovery (Figure 3A) and a significant ($p = 7 \times 10^{-8}$) increase in apoptosis (Figure 3B) of LCMV-specific CTLs in Spi6 KO mice. In Spi6 KO x GrB KO mice, the number (Figure 3A) and proportion of CTLs undergoing apoptosis (Figure 3B) were the same as B6 controls. Thus, the absence of GrB corrected the deficit in CTL survival caused by Spi6 deficiency and returned the level of LCMV-specific CTLs to wild-type levels. GrB KO mice have partially reduced expression of the linked granzymes C and F (Revell et al., 2005), which may also contribute to the correction of CTL survival in Spi6 KO mice. However, Spi6 directly inhibits purified GrB in vitro (Sun et al., 1997) (data not shown) and protects perforin-loaded cells from purified GrB (Phillips et al., 2004). Therefore, we conclude that protection from GrB is at least in part a physiological mechanism by which Spi6 ensures the survival of CTLs after infection.

Spi6 can protect DCs from granule-mediated killing by CTLs (Medema et al., 2001a), and so reduced expansion of CTLs caused by defective priming may occur in Spi6 KO mice. We determined whether the requirement for Spi6 for CTL survival was cell autonomous. Spi6 KO mice were crossed with C57BL/6 P14 transgenic mice, which express a T cell receptor (TCR) specific for the gp33 peptide antigen of LCMV in the context of H-2D^b

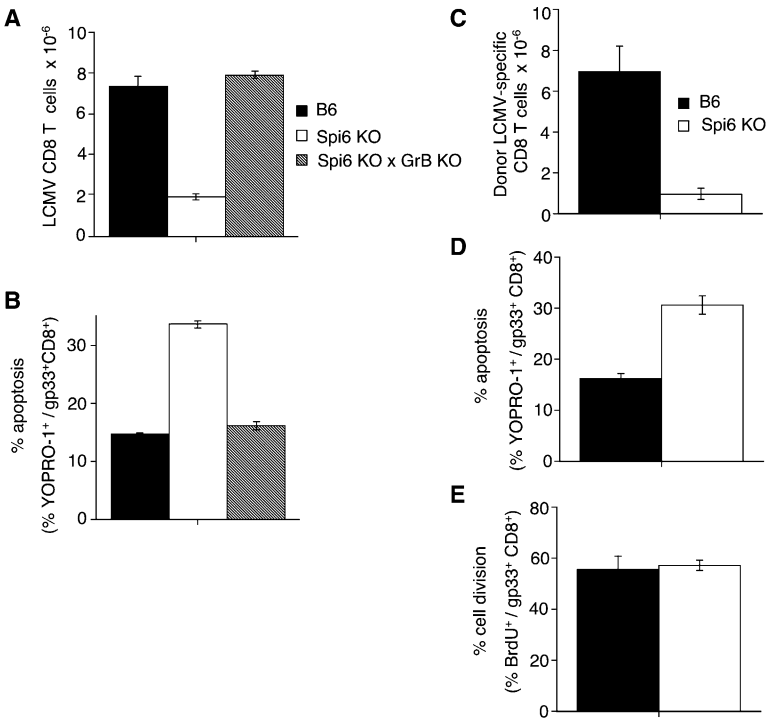

Figure 3. Effect of Spi6 on CTLs Is Dependent on GrB

B6 and Spi6 KO mice were infected with LCMV Armstrong (2×10^5 pfu/mouse i.p.), and then after 8 days the (A) mean number of $gp33^+$ $CD8^+$ spleen cells and (B) mean percentage YOPRO-1^+ of $gp33^+$ $CD8^+$ cells were determined. P14 CD8 T cells (10^4) were purified from B6 or Spi6 KO mice ($CD45.2^+$) and adoptively transferred to B6 $CD45.1^+$ congenic mice, and then the (C) mean number of donor LCMV-specific ($CD45.2^+$ $gp33^+$ $CD8^+$) and (D) mean percentage of YOPRO-1^+ donor cells determined. Recipient mice were infected and fed BrdU for 8 days, and then the (E) mean percentage $BrdU^+$ donor cells determined. All means are ± SEM (n = 5 mice).

(Pircher et al., 1990). Naive P14 CD8 T cells (>90% pure) from Spi6 KO and B6 mice ($CD45.2^+$) were adoptively transferred into B6 CD45.1 congenic recipients, which were then infected with LCMV. After 8 days, the number of donor LCMV-specific CTLs ($CD45.2^+$) from Spi6 KO mice was diminished by 7-fold ($p = 3 \times 10^{-8}$) compared to B6 P14 donor cells (Figure 3C). Therefore, the requirement for Spi6 in CTLs is cell intrinsic. As for the whole animal experiments, there was a significant (p = 0.04) increase in the proportion of donor Spi6 KO CTLs undergoing apoptosis after adoptive transfer to wild-type recipients (Figure 3D). There was, however, no significant difference (p = 0.2) in the rate of cell division of donor Spi6 CD8 T cells compared to B6 controls over the 8 day expansion period after LCMV infection (Figure 3E). Thus, the diminished recovery of donor Spi6 KO CTLs is a result of impaired survival due to increased apoptosis rather than impaired expansion.

Impaired CTL Immunity to Virus in Spi6-Deficient Mice

CTLs are critical for immunity to virus, and so we examined the ability of Spi6 KO mice to mount a CTL response and clear LCMV. We examined the requirement for Spi6 in immunity to high doses (10^6 pfu) of the clone 13 variant of LCMV Armstrong, which has previously revealed deficiencies in CTL immunity to virus (Ahmed et al., 1984; Matloubian et al., 1999). Ex vivo CTL activity was measured in standard ^{51}Cr-release assays with gp33 pulsed targets. We observed a consistent deficit in LCMV-specific CTL activity in Spi6 KO mice over the course of clone 13 infection (Figure 4A). For example, on day 10, there was a 6-fold decrease ($p = 2 \times 10^{-4}$) in CTL activity in Spi6 KO mice (2.3% ± 0.7% specific lysis, n = 5 mice; Splenocyte/Target ratio [S/T] = 50:1) compared to B6 controls (13.6% ± 1.7% specific lysis, n = 5 mice; S/T = 50:1).

The deficit in CTL activity resulted in correspondingly defective clearance of clone 13 (Figure 4B). Plaque assays revealed that the titer of LCMV was about 10 times higher in Spi6 KO mice on day 6 (p = 0.0067), day 8 (p = 0.0043), and day 10 (p = 0.002) after infection. The lower number of LCMV-specific CTLs in Spi6 KO mice, rather than any intrinsic defect in killing, results in impaired CTLs activity. This was because, on day 8 after infection with LCMV, although we observed defective CTL activity in Spi6 KO mice as expected (Figure 4C), when specific lysis was normalized for the number of input LCMV-specific CTLs ($gp33^+$ $CD8^+$ cells), there was no significant (p = 0.63; CTL/Target ratio = 1.0) difference between Spi6 KO mice and B6 controls (Figure 4D).

We determined whether the impaired CTL-mediated clearance of clone 13 LCMV in Spi6 KO mice was due to a cell-autonomous defective in CD8 T cells. Naive P14 CD8 T cells from Spi6 KO and B6 mice were adoptively transferred into B6 recipients, which were then infected with clone 13 LCMV. On day 6, gp33-specific CTL activity was 5 times higher (S/T = 50:1; $p = 6 \times 10^{-4}$) in B6 recipients of B6 P14 CD8 T cells (Figure 4E) than the endogenous level in similarly infected B6 mice on day 6 (Figure 4A). Therefore, the majority of LCMV-specific CTLs in B6 recipients are derived from donor P14 CD8 T cells on day 6 after infection with clone 13 LCMV. There was significantly lower LCMV-specific CTL activity derived from Spi6 KO P14 CD8 T cell donor cells (Figure 4E). For example, there was a 5-fold decrease ($p = 1 \times 10^{-4}$) in donor Spi6 KO CTLs (3.6% ± 1.2% specific lysis, n = 5 mice; S/T = 5.6) compared to donor B6 controls (19.1% ± 1.9% specific lysis, n = 5 mice; S/T = 5.6). The defect in donor Spi6 KO CTLs specific for clone 13 resulted in defective clearance, as evidenced by a

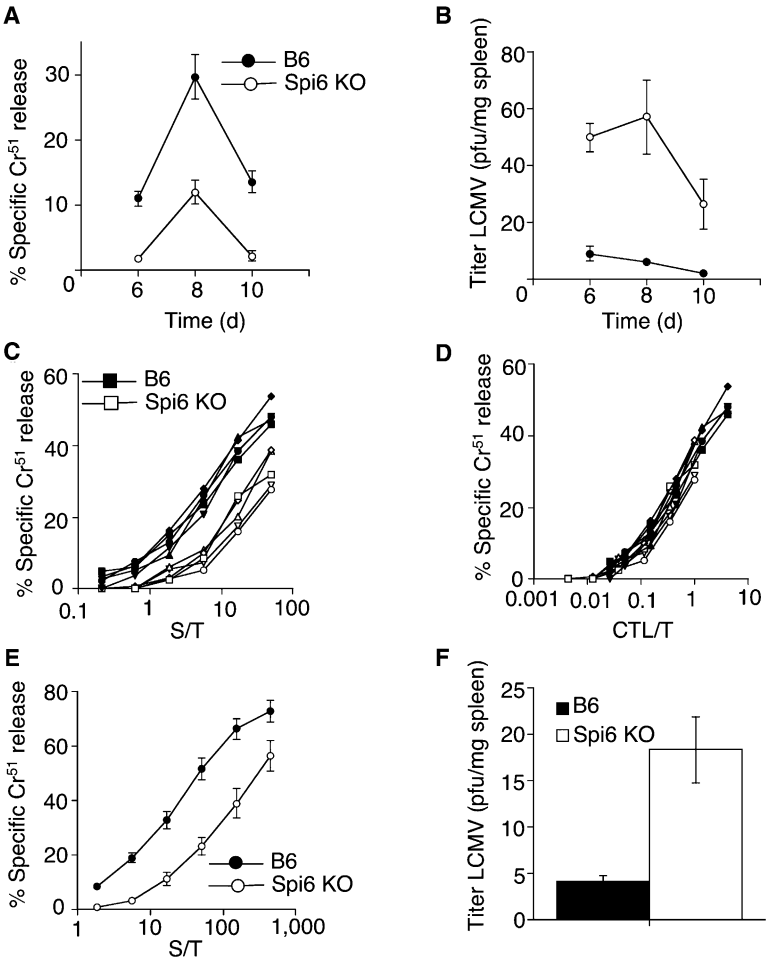

Figure 4. Impaired CTL Immunity in Spi6 KO Mice

B6 and Spi6 KO mice were infected with LCMV clone 13 (10^6 pfu/mouse i.v.).

(A) Ex vivo anti-LCMV CTL activity was measured by determining % specific lysis of ^{51}Cr-labeled RMA target (T) cells (H-2^b) pulsed with LCMV gp33 peptide (mean of four determinations) by splenocytes (S) from infected mice. Data are the mean % specific Cr^{51}-release ± SEM (n = 5 mice) at a S/T ratio of 50:1.

(B) Titer of clone 13 LCMV determined in spleen homogenates by plaque assay on Vero cells. Data are the mean titer expressed as pfu per mg spleen ± SEM (n = 5 mice).

(C) Ex vivo anti-LCMV CTL activity determined as % specific lysis gp33-pulsed targets on day 8 after infection with LCMV Armstrong (2×10^5 pfu/mouse i.p.). Data are the mean of four determinations of % specific Cr^{51}-release from individual mice over a range of S/T ratios.

(D) Ex vivo anti-LCMV CTL activity was measured as in (A) at various ratios of gp33$^+$ CD8$^+$ (CTL) to targets (T). P14 CD8 T cells (10^4) were purified from B6 or Spi6 KO mice and adoptively transferred to wild-type B6 mice, then infected with LCMV clone 13 (10^6 pfu/mouse i.v.).

(E) Ex vivo anti-LCMV CTL activity determined as % specific lysis gp33-pulsed targets on day 6 after infection. Data are the mean % specific Cr^{51}-release ± SEM (n = 5 mice) over a range of S/T ratios.

(F) Titer of clone 13 LCMV from the spleens of recipient mice on day 6 after infection. Data are the mean titer expressed as pfu per mg spleen ± SEM (n = 5 mice).

4-fold increase in titer (p = 0.004) (Figure 4F). We conclude that deficiency in Spi6 directly results in the defective survival of CD8 T cells leading to impaired CTL activity and clearance of LCMV.

Spi6 Suppresses Cytoplasmic GrB in CTLs

It has been suggested that leakage of GrB from granules into the cytoplasm can lead to the activation-induced cell death of cytotoxic lymphocytes (Ida et al., 2003). Therefore, we examined whether Spi6 was required to protect against this pathway of death in CTLs. LCMV-specific CTLs were generated by culturing spleen cells from P14 mice with gp33 for 2 days in vitro (>90% of viable cells) (Phillips et al., 2004), then subjected to subcellular fractionation (Sun et al., 1996). Western blots after reducing SDS-PAGE revealed Spi6 in the cytoplasm but not granule fractions of B6 CTLs (Figure 5A). The subcellular localization of Spi6 to the cytoplasm is consistent with the lack of a secretory signal, as has been observed for its human homolog PI9 in CTLs (Sun et al., 1996). As expected, Spi6 was absent from Spi6 KO CTLs. A 67 kDa form of Spi6 was present in the cytosol of B6 CTLs but not GrB KO CTLs. Probing Spi6 KO CTLs with anti-GrB antiserum failed to detect the 67 kDa form, confirming it as a SDS-stable complex between Spi6 and GrB (Figure 5A).

Measurement of GrA activity by a specific substrate (Odake et al., 1991) served as a marker for granule integrity independent of GrB. As expected, the specific activity of GrA was about 4 times higher ($p = 3 \times 10^{-4}$) in granule compared to the cytosol fraction of GrB KO CTLs (Figure 5B). Enzyme assays revealed significantly (p = 0.004) increased specific activity of GrB in the cytoplasm of Spi6 KO compared to B6 CTLs (Figure 5B). Consistent with its specificity as a serpin of GrB (Sun et al., 1997), we conclude that Spi6 inhibits cytoplasmic GrB through the formation of covalent complexes. We observed lower specific activity of the caspase 3 executioner protease (Budihardjo et al., 1999) in the cytoplasm of GrB KO CTLs ($p = 2 \times 10^{-4}$), indicating that endogenous GrB induces apoptosis of CTLs (Figure 5B). There was a corresponding increase in the specific activity of caspase 3 in the cytoplasm of Spi6 KO CTLs (p = 0.002) (Figure 5B). Since substrates cleaved by GrB that trigger apoptosis are located in the cytoplasm (Russell and Ley, 2002), we conclude that the increased apoptosis in Spi6 KO CTLs is at least in part due to increased GrB activity in the cytoplasm.

Inhibition of GrB by Spi6 Ensures the Integrity of Cytotoxic Granules

In addition to suppressing the activity of cytoplasmic GrB, Spi6 also ensured the integrity of cytotoxic granules. Subcellular fractionation studies revealed a decrease in the specific activity of both GrB and GrA in the granules and an increase in the cytoplasm of Spi6

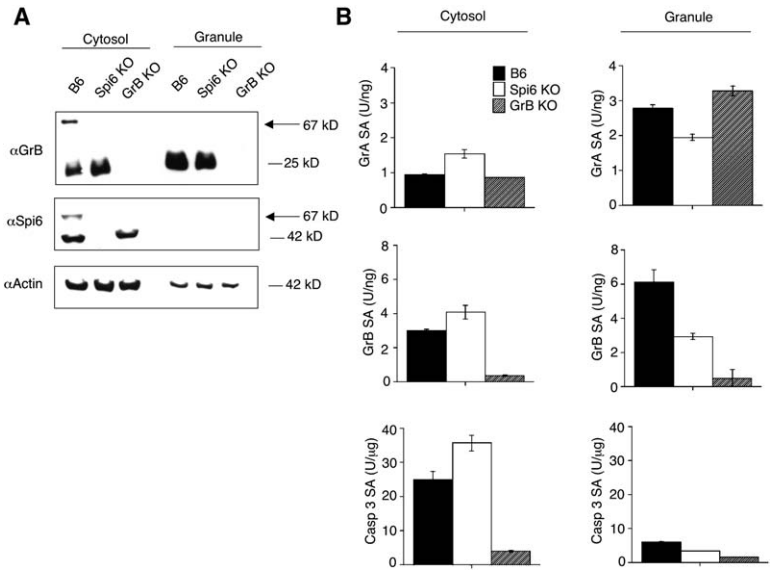

Figure 5. Spi6 Deficiency Increases Cytoplasmic GrB and Apoptosis in CTLs

(A) Western blots of reducing SDS-PAGE from the cytosol and granules of wild-type B6, Spi6 KO, or GrB KO P14 CTLs.

(B) Mean specific activity (SA) ± SEM of GrA, GrB, and caspase 3 (casp 3) from CTLs (n = 4 mice).

KO CTLs (Figure 5B). However, it is possible that the rupture of granules during the subcellular fractionation procedure may result in granzyme activity in cytosolic fractions. Therefore, we used confocal immunofluorescence microscopy (CIM) to examine the effect of Spi6 deficiency on granule stability in intact CTLs. The frequency of Spi6 KO CTLs devoid of GrB^+ granules (>0.1 μm in diameter) was about 3 times greater than for B6 CTLs ($p = 0.03$), resulting in a 2-fold decrease ($p = 0.03$) in the mean number of GrB^+ granules per Spi6 KO CTL (Figures 6A and 6B). Furthermore, there was a corresponding increase in GrB staining in the cytoplasm of Spi6 KO CTLs. (Figure 6A). We also observed a loss of GrB^+ Pfn^+ granules in Spi6 KO CTLs (Figure 6C), implying that Spi6 prevents GrB leakage into the cytoplasm by ensuring general granule integrity.

Granule vesicles from murine CTLs are composed of several clustered membrane bound electron-dense cores with a diameter of about 100 nm (Peters et al., 1991). We used transmission electron microscopy (TEM) to determine the number of electron-dense cores in 0.1 μm thick sections (n = 300) of Spi6 P14 CTLs, and after extrapolation based on the cell volume, the total number per CTL (Peters et al., 1991). Spi6 KO CTLs contained 7 times ($p = 0.02$) fewer electron-dense cores (14 ± 4) than B6 CTLs (102 ± 20), although there was no significant difference ($p = 0.5$) in the mean diameter of the electron-dense cores (B6 120 ± 30 nm, Spi6 KO 125 ± 26 nm). Representative examples of electron micrographs of B6 and Spi6 CTLs are shown in Figure 6D. The defect in the number of granule cores in Spi6 CTLs is consistent with the decrease in the number of $GrnB^+$ Pfn^+ granules revealed by CIM (Figure 6C). As expected, about 40% of the electron-dense granule cores in wild-type CTLs were found in clusters of three or more, indicating the presence of a granule vesicle (Peters et al., 1991). However, we did not observe any clustering of the electron-dense granule cores into granule structures in Spi6 KO CTLs (Figure 6D). Thus, as well as reducing the number of core vesicles, Spi6 deficiency also seems to disrupt the organization of these cores into granule vesicles.

The defect in granule integrity was GrB dependent because inhibition of GrB by Z-AAD-CMK (Gong et al., 1999) gave a significant ($p = 2 \times 10^{-6}$) increase in the number of granules in Spi6 KO CTLs (Figure 6E). As we observed before (Figure 5B), there was a 2-fold decrease ($p = 9 \times 10^{-5}$) in granule-associated GrA-specific activity in Spi6 KO CTLs compared to B6 control CTLs (Figure 6F). The defect in Spi6 KO CTLs could be corrected in Spi6 KO x GrB KO CTLs, as indicated by a complete rescue of GrA-specific activity in granules (Figure 6E). We conclude that the increased GrB activity in the cytoplasm of Spi6 KO is the catalyst for granule breakdown and amplification of GrB-mediated effects. The significant ($p = 0.004$) increase in granule-specific GrA activity in GrB KO CTLs over the level in B6 CTLs (Figure 6F) suggests that GrB-catalyzed breakdown of granules may occur in wild-type CTLs. This is supported by the increase ($p = 0.01$) in the mean number of electron-dense granule cores in GrB CTLs (150 ± 67, n = 300 cell sections) compared to B6 CTLs (102 ± 20) (Figure S1).

Discussion

Studies that describe the expression of PI9 and Spi6 in leukocytes and inflammatory sites have led to the suggestion that serpins protect against GrB during immune reactions (Bladergroen et al., 2001; Hirst et al., 2003). These observations have been complemented by ectopic overexpression studies that show the potential for Spi6 in protecting from GrB-mediated death (Medema et al., 2001b; Phillips et al., 2004). However, artificially high levels of Spi6 expression were required for cyto-protection (Medema et al., 2001b; Phillips et al., 2004), and so the physiological requirement for Spi6 was not tested. To address this issue, we generated Spi6 KO mice and examined the viability, function, and structure of CTLs.

We show that Spi6 protects CTLs from their own GrB by suppressing activity in the cytoplasm. The breakdown of cytotoxic granules by GrB in the cytoplasm was unexpected, but likely amplifies the release of GrB and induction of apoptosis (Figure 7). How the increased

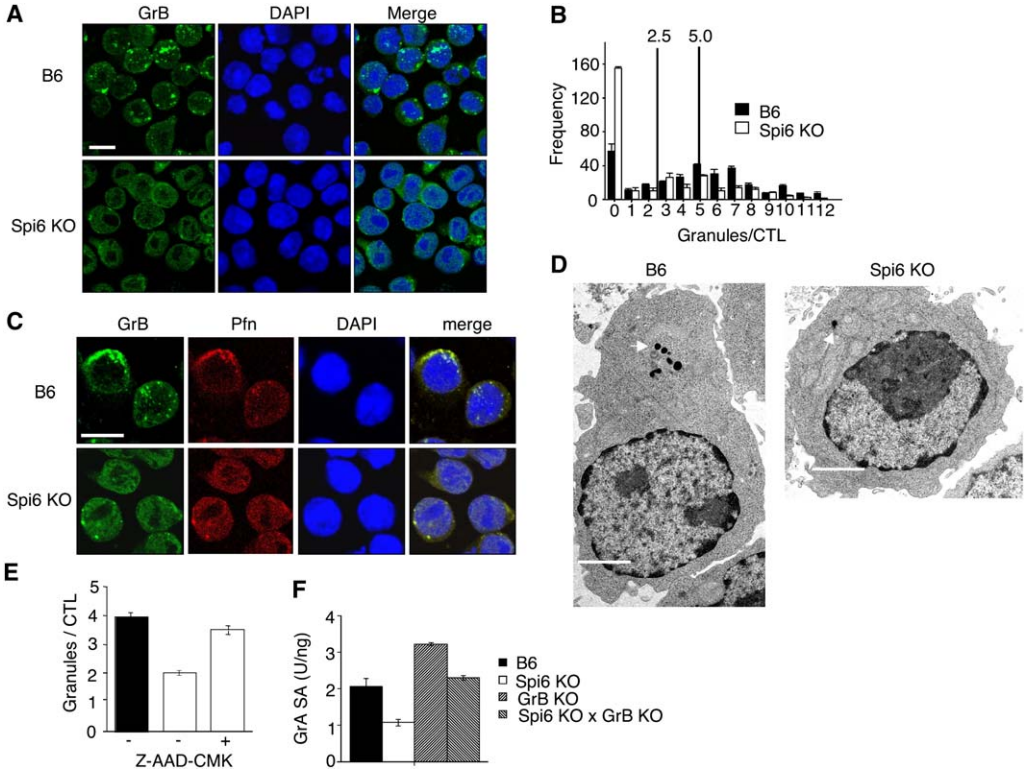

Figure 6. Spi6 Deficiency Destabilizes Cytotoxic Granules

(A) Visualization of GrB$^+$ granules in P14 CTLs by CIM. Nuclei were stained by DAPI. Scale bar equals 5 μm.

(B) Distribution of the number of GrB$^+$ granules (diameter > 0.1 μm) per P14 CTL (n = 300 cells) measured by CIM. Mean values are indicated.

(C) Visualization of GrB$^+$ Pfn$^+$ granules in P14 CTLs by CIM. Scale bar equals 5 μm.

(D) TEM (×4060 magnification) of P14 CTLs from B6 and Spi6 KO mice. The arrow indicates electron dense granule cores (a cluster of seven in B6 CTLs, one in Spi6 KO CTL). Scale bar equals 2 μm.

(E) Effect of Z-AAD (OMe)-CMK on the number of GrB$^+$ granules (diameter > 0.1 μm) per P14 CTL ± SEM (n = 300 cells) measured by CIM.

(F) Mean SA of GrA in the granule fractions of P14 CTLs ± SEM (n = 4 mice).

cytoplasmic GrB in Spi6 KO CTLs leads to granule breakdown is unclear but could involve direct targets on the granule or could be a more indirect consequence of biochemical events triggered by cytoplasmic GrB (Russell and Ley, 2002). The potency of GrB as a CTL effector molecule likely explains why less than a 2-fold increase in cytoplasmic GrB activity (Figure 5B) results in the near complete loss (10%–20% of wild-type level) of Spi6 KO CTLs in vivo (Figure 2). GrB kills by triggering multiple apoptotic pathways involving caspases and mitochondrial dysfunction in addition to DNA fragmentation (Russell and Ley, 2002). By directly targeting GrB, Spi6 can potentially suppress all of the toxic effects of a CTL effector molecule. It has been suggested that inhibition of another CTL effector molecule—Pfn—by cathepsin B also protects CTLs from self-inflicted damage (Balaji et al., 2002). However, in vivo studies indicate that cathepsin B induces the apoptosis of CTLs (Liu et al.,

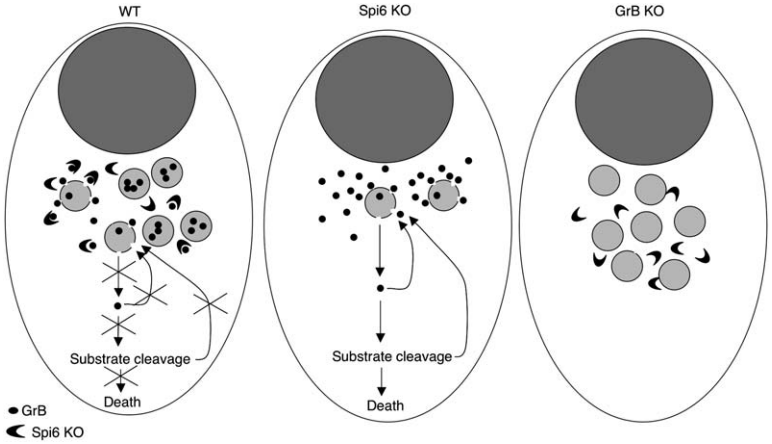

Figure 7. Spi6 Inhibits GrB-Mediated Apoptosis of CTLs

Spi6 inhibits GrB in the cytoplasm of wild-type (wt) CTLs (nucleus in dark gray) through the formation of an irreversible complex. The inhibition of GrB (crossed line) blocks the cleavage and activation of target enzymes and the induction of death (crossed line). The breakdown of granule integrity and the release of more GrB, either directly by cytoplasmic GrB or by downstream targets, is thus inhibited by Spi6. Cytoplasmic GrB is not blocked in Spi6 KO CTLs and so there is increased death. A positive feedback loop whereby GrB release into the cytoplasm catalyzes further leakage from granules then ensues because GrB causes granule damage. There is no GrB-mediated breakdown of granules in GrB KO CTLs resulting in increased granule number and decreased death.

2004). Thus, Spi6 seems to be unique as an inhibitor of self-inflicted damage by CTLs that has been validated in vivo.

The expression of peptide antigen/MHC (pMHC) on CTLs after infection by virus (Hanon et al., 2000) or after reexpression of target cell pMHC after killing (Huang et al., 1999) can lead to fratricide. However, our findings with Spi6 are consistent with the induction of suicide by endogenous GrB in the cytoplasm of CTLs (Opferman et al., 2001) and NK cells (Ida et al., 2003). In our studies, we find that Spi6 KO CTLs undergo increased apoptosis because their own granules release GrB into the cytoplasm. In addition, GrB-mediated granule breakdown likely also occurs in wild-type CTLs (Figure 6E). Although our studies do not rule out the possibility that fratricide of CTLs is a physiological mechanism for clearing CTLs in vivo, they do show that GrB-mediated death may occur in wild-type CTLs without fratricide.

Cytotoxic T cells from patients with Chediak-Higashi (Baetz et al., 1995; Barbosa et al., 1996; Stinchcombe et al., 2000; Ward et al., 2000) and Griscelli (Menasche et al., 2000) syndromes and from the relevant mouse models (Beige [Perou et al., 1996a, 1996b] and Ashen [Wilson et al., 2000]) have defective granule killing. This is due to mutations that disrupt the secretion of granzymes but do not diminish the number of cytotoxic granules. Therefore, the decrease in the number of cytotoxic granules we observed in Spi6 KO CTLs was unexpected. Those ex vivo Spi6 KO CTLs we could recover were not impaired in their ability to kill (Figure 4D). This implies that "granule-less" Spi6 KO CTLs are likely to be very short lived in vivo, presumably because of their increased susceptibility to apoptosis. CIM revealed that Spi6 KO harbor fewer GrB^+ Pfn^+ granules (Figures 6A–6C), which was confirmed when TEM revealed a corresponding deficit in the number of electron-dense granule cores (Figure 6D). In wild-type mice, granule vesicles are composed of clustered membrane bound, electron-dense cores (Peters et al., 1991). We did not observe any clustering of electron-dense cores in Spi6 KO CTLs, suggesting a disruption in both the stability and organization of core vesicles within granule vesicles. That GrB is the cause of granule instability CTLs is demonstrated by our observations that GrB synthetic inhibitors (Figure 6E) and genetic deficiency (Figure 6F) can restore the integrity of granules in Spi6 KO CTLs.

Given the role of Spi6 in protecting CTLs from self-inflicted damage, one might predict that some human CTLs immunodeficiencies may potentially also be due to mutations in the PI9 human homolog. Ectopic expression of PI9 can enhance human CTL killing (Hirst et al., 2003), and so suppression of cytoplasmic GrB may have therapeutic value in rescuing defective CTL function in chronic viral infections (McMichael, 1998). PI9 is also upregulated in cells in immune-privileged sites (Bladergroen et al., 2001) and so may also protect against inflammatory disease by suppressing GrB.

Experimental Procedures

Mice

Spi6 cDNA (Phillips et al., 2004) was used to clone mouse genomic DNA containing the Spi6 locus (*Serpin b9*) from a bacterial artificial chromosome (BAC) library (129/Sv strain RPCI-22, Res Gen). The 5′ homology region (4.1 kb *Sac II-Kpn I* fragment) and the 3′ homology region (3.6 kb *Not I-Xho I* fragment) were cloned on either side of the *neo* gene (1.8 kb *Kpn I-Not I* fragment) flanked by *loxP* recombination sites (Kuhn et al., 1995). C57BL/6 ES cells (Ware et al., 2003) were transfected with targeting vector (50 μg). DNA from G418-resistant clones was digested with *Spe I* and *Hind III* and hybridized with probes to detect wild-type (5′, 7.6 kb; 3′, 9.8 kb) and mutant (5′, 6.9 kb; 3′, 5.8 kb) alleles. ES cells from targeted clones (2/398) were transfected (30 μg) with *Cre* (pBS185, Invitrogen), and excision of the *neo* after *loxP* site-specific recombination was detected by the presence of a 5.8 kb band after blotting with 5′-probe (Kuhn et al., 1995). C57BL/6 ES cells were microinjected into BALB/c blastocysts to produce chimeric mice, which were then backcrossed against wild-type C57BL/6 mice (Jackson Laboratory). C57BL/6 $Spi6^{+/-}$ mice (from two independently targeted ES cell clones) were intercrossed to generate C57BL/6 $Spi6^{-/-}$ mice (Spi6 KO mice), which were born at a Mendelian frequency.

Spi6 KO mice were crossed with $GrB^{-/-}$ C57BL/6 (GrB KO) (Heusel et al., 1994) (Jackson Laboratory) to generate C57BL/6 $Spi6^{-/-}$ $GrB^{-/-}$ mice (Spi6 KO x GrB KO). C57BL/6 *P14* $TCR^{+/-}$ transgenic mice (B6 P14 mice) (Pircher et al., 1990) were crossed with either Spi6 KO or Spi6 KO x GrB KO mice to produce C57BL/6 Spi6 KO *P14* $TCR^{+/-}$ (Spi6 KO P14 mice) or C57BL/6 Spi6 KO x GrB KO *P14* $TCR^{+/-}$ (Spi6 KO x GrB KO P14 mice) transgenic mice. C57BL/6 CD45.1 congenic mice were purchased from Taconic. All mice were maintained and bred under standard specific pathogen-free (SPF) conditions. All experiments with mice were performed in compliance with the University of Chicago Institutional Animal Care and Use Committee regulations.

Real-Time PCR

Leukocytes were purified (>95%) for real-time PCR analysis from splenocytes by magnetic beads conjugated to phenotypic markers (Miltenyi Biotec). Macrophages were generated from bone marrow cultured in media containing macrophage colony stimulating factor by standard protocols (Coligan et al., 1995). DCs (immature) were generated from bone marrow, as described before (Lutz et al., 1999). Neutrophils were recovered from the peritoneum by lavage 4 hr after i.p. injection with 15% glycogen (1 ml), as described before (Lopez-Boado et al., 2004). RNA was extracted from purified cell populations by TriZOL Reagent (Invitrogen), and then cDNA was generated by Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) (Medhurst et al., 2000). Primer and probe sequences for *Spi6, Spi3,* and *cyclophilin A* are described in Supplemental Experimental Procedures online and were designed by Primer Express software (PE Applied Biosystems). The data were calculated as the ratio of candidate RNA expression/amount of *cyclophilin A*, as before (Liu et al., 2004).

Infections

Mice were infected by either i.p. injection (2×10^5 pfu) of LCMV Armstrong (Phillips et al., 2004) or i.v. injection (10^6 pfu) of the clone 13 variant of LCMV Armstrong (Ahmed et al., 1984). For LM, mice were infected by i.v. injection of DPL-1942 (10^5 cfu), which has been engineered to express ovalbumin and in C57BL/6 mice generates the H-$2K^b$-restricted peptide epitope (SIINFEKL: OVA) (Pope et al., 2001). LCMV was titered on monolayers of Vero cells (Ahmed et al., 1984).

Flow Cytometry

The following mAbs were used (BD-Pharmingen): anti-CD8α (allophycocyanin [APC]-labeled), anti-CD45.2-fluorescein isothiocynate [FITC] or R-phycoerythrin [PE], anti-$CD11b^+$-FITC, anti-F4/80-PE, anti-DX5-PE, anti-B220-FITC, anti-BrdU-FITC (IgG_1) and IgG_1 isotype control-FITC. H-$2D^b$-tetramers with gp 33 [KAVYNFATM] (Phillips et al., 2004) or H-$2K^b$-tetramers with OVA were labeled with streptavidin-PE (Beckman Coulter). Thymocytes, PBLs and splenocytes were prepared and stained with tetramers and mAbs as before (Phillips et al., 2004). Apoptosis of live cells (propidium iodide-negative) was measured with YOPRO-1 dye (green fluorescence) (Molecular Probes), as before (Opferman et al., 2001). Intracellular staining with anti-BrdU mAb was used to determine BrdU incorporation in $CD45.2^+$ $gp33^+$ $CD8^+$ cells, as before (Phillips et al., 2004).

Adoptive Transfer

Naive $CD8^+$ cells were purified (>90%) from the spleens of P14 mice ($CD45.2^+$) by positively sorting with anti-CD8 magnetic beads (Miltenyi Biotec) and adoptively transferred (10^4) by i.v. injection into C57BL/6 CD45.1 mice and after 1 day infected with LCMV. To measure P14 CD8 T cell division, recipients were given BrdU in their drinking water (0.8 mg/ml) for 8 days after LCMV infection (Phillips et al., 2004).

Ex Vivo CTL Assays

To measure ex vivo CTL activity after LCMV infection, RMA targets were pulsed with gp33 (10^{-7} M) for 1 hr and labeled with $^{51}Cr-$, then incubated with viable splenic leukocytes over a range of ratios in quadruplicate. The percentage specific lysis was determined after 4 hr as follows: % specific release = (specific release − spontaneous release)/(maximum release × spontaneous release) × 100 (Opferman et al., 2001). The percentage specific lysis of RMA cells in the absence of gp33 was <10% and the spontaneous release was <10% of the maximum release.

Subcellular Fractionation of CTLs

Spleen cells (10^6/ml) from B6 P14 mice, Spi6 KO P14 mice, or Spi6 KO x GrB KO P14 mice were cultured with LCMV gp33 peptide [KAVYNFATM] (10^{-6} M) and IL-2 (10 U/ml) for 2 days to generate CTLs (Phillips et al., 2004). CTLs were lysed by sonication in hypotonic buffer (50 mM PIPES, 50 mM KCL, 5 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT [pH 7.6]), then centrifuged at 3000 × g for 20 min to remove nuclei, then 15,000 × g for 30 min to give cytosol (supernatant) and granule (pellet) fractions (Sun et al., 1996). The granule pellet was resuspended in 1% Triton X-100, 10 mM Tris.HCl, 150 mM NaCl (pH 7.6) for 30 min on ice.

Western Blotting

Antiserum specific to a Spi6 peptide (amino acids 35–47) ([C]RKLNKPDRKYSLR) was raised in rabbits by standard procedures (Coligan et al., 1995). In brief, two rabbits were immunized with the peptide conjugated to KLH and then boosted twice with immunogen over a period of 3 months. Anti-Spi6 antibodies were affinity purified on columns of immunizing peptide, eluted in 3M KSCN, and then dialyzed against PBS. Protein (50 μg) was resolved by reducing SDS-PAGE, then immunoblotted and probed with anti-Spi6 antiserum (7 μg/ml) and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (2 μg/ml) (Sigma-Aldrich), then visualized by chemiluminescence (ECL-kit, Amersham). Blots were also probed with goat anti-mouse GrB (0.2 μg/ml) (R&D Systems) and anti-goat IgG HRP (2 μg/ml) (Sigma-Aldrich) or with anti-actin monoclonal antibody clone ACTN05 (0.5 μg/ml) (Sigma Aldrich) and anti-mouse IgG-HRP (2 μg/ml) (Sigma-Aldrich).

Protease Assays

Colorimetric assays for GrB were performed in reaction buffer with Ac-IEPD-*p*NA at 0.2 mM (Liu et al., 2003) and GrA with BLT (N^a-benzyloxycarbonyl-L-lysine thiobenzyl ester) at 0.2 mM (Odake et al., 1991) and Ellman's reagent at 1.76 mM in reaction buffer (100 mM Tris-HCl) at 30ºC. Assays for caspase 3 were performed in reaction buffer (10 mM PIPES [pH 7.4], 8 mM DTT, 2 mM EDTA, 0.1% CHAPS) at 30ºC with Ac-DEVD-*p*NA (Calbiochem) at 0.2 mM (Liu et al., 2003). Specific activity was determined by normalizing for the amount of protein. Units of activity were as defined before (Liu et al., 2003). To inhibit GrB (Gong et al., 1999; Odake et al., 1991), Z-AAD (OMe)-CMK (12.5 μM; Sigma Aldrich) was added for the duration of 2 day cultures. Under these conditions, we observed 100% inhibition of GrB in both the granule and cytoplasmic fractions.

CIM

P14 CTLs were seeded on wells of poly-L-Lysine-coated slides, then fixed and permeabilized in acetone/methanol and blocked with 10% normal horse serum in PBS for 60 min at 20ºC. P14 CTLs were stained with anti-murine GrB Ab (1:200; R&D Systems) and anti-murine Pfn P1-8 mAb (1:100; Kamiya Biomedical Co) for 60 min at 20ºC in 2% BSA/PBS, then biotin-conjugated mouse anti-Rat mAb (1:100; BD Pharmingen) and FITC conjugated rabbit anti-goat antibody (1:200; Molecular Probes) for 60 min at 20ºC. After washing with PBS, cells were further incubated with streptavidin-PE (1 μg/ml; BD Pharmingen) and DAPI (Sigma Aldrich), washed, and mounted in Vectashield (Vector Labs). Cells were examined with a Leica SP2 AOBS spectral laser scanning confocal microscope operated with the software LCS 2.5v1347. The mean number of granules was determined from the counting of multiple cell layers (100 cells in 3 separate counts, n = 300) with a granule defined as point of staining with a diameter >0.1 μm.

TEM

Cells were stained with uranyl acetate/lead citrate, as described in Supplemental Experimental Procedures. An electron microscope (FEI Tecnai F30) at 300 kV was used for examination. Electron-dense core vesicles were counted in 64 μm^2 cell areas (n = 300), and then the total mean number per cell was determined assuming a mean cell volume of 523 μm^3 (mean number per 64 μm^2 × 8.2 = mean total number per cell).

Statistics

The significance of difference was measured using two-tailed Student's t tests.

Supplemental Data

Supplemental Data include one figure, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/24/4/451/DC1/.

Acknowledgments

We would like to thank R. Welsh for high titer stocks of LCMV Armstrong and Y. Chen of the University of Chicago Electron Microscopy Core facility for TEM. We thank G. Franzoso and S. Byrne for useful comments on the paper and F. Tang for help with Western blots. Supported by NIH grant AI45108 to P.G.A.-R.

Received: September 23, 2005
Revised: February 1, 2006
Accepted: February 3, 2006
Published: April 18, 2006

References

Ahmed, R., Salmi, A., Butler, L.D., Chiller, J.M., and Oldstone, M.B.A. (1984). Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. J. Exp. Med. *160*, 521–540.

Badovinac, V.P., Tvinnereim, A.R., and Harty, J.T. (2000). Regulation of antigen-specific T cell homeostasis by perforin and interferon-γ. Science *290*, 1354–1357.

Badovinac, V.P., Hamilton, S.E., and Harty, J.T. (2003). Viral infection results in massive $CD8^+$ T cell expansion and mortality in vaccinated perforin-deficient mice. Immunity *18*, 463–474.

Baetz, K., Isaaz, S., and Griffiths, G.M. (1995). Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. J. Immunol. *154*, 6122–6131.

Balaji, K.N., Schaschke, N., Machleidt, W., Catalfamo, M., and Henkart, P.A. (2002). Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. J. Exp. Med. *196*, 493–503.

Barbosa, M.D., Nguyen, Q.A., Tchernev, V.T., Ashley, J.A., Detter, J.C., Blaydes, S.M., Brandt, S.J., Chotai, D., Hodgman, C., Solari, R.C., and Kingsmore, S.F. (1996). Identification of the homologous beige and Chediak-Higashi syndrome genes. Nature *382*, 262–265.

Bladergroen, B.A., Strik, M.C.M., Bovenschen, N., van Berkum, O., Scheffer, G.L., Meijer, C.J.L.M., Hack, C.E., and Kummer, J.A. (2001). The granzyme B inhibitor, protease inhibitor *9*, is mainly expressed by dendritic cells and at immune-privileged sites. J. Immunol. *166*, 3218–3225.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. *15*, 269–290.

Coligan, J.E., Kruisbeek, A.M., Margulis, D.H., Shevach, E.M., and Strober, W. (1995). Current Protocols in Immunology, *Vol 1* (New York: John Wiley and Sons).

Gong, B., Chen, Q., Endlich, B., Mazumder, S., and Almasan, A. (1999). Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases. Cell Growth Differ. *10*, 491–502.

Hanon, E., Stinchcombe, J.C., Saito, M., Asquith, B.E., Taylor, G.P., Tanaka, Y., Weber, J.N., Griffiths, G.M., and Bangham, C.R. (2000). Fratricide among CD8(+) T lymphocytes naturally infected with human T cell lymphotropic virus type I. Immunity *13*, 657–664.

Heusel, J.W., Wesselschmidt, R.L., Shresta, S., Russell, J.H., and Ley, T.J. (1994). Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell *76*, 977–987.

Hirst, C.E., Buzza, M.S., Bird, C.H., Warren, H.S., Cameron, P.U., Zhang, M., Ashton-Rickardt, P.G., and Bird, P.I. (2003). The intracellular granzyme B inhibitor, proteinase inhibitor *9*, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. J. Immunol. *170*, 805–815.

Huang, J.-F., Yang, Y., Sepulveda, H., Shi, W., Hwang, I., Peterson, P.A., Jackson, M.R., Sprent, J., and Cai, Z. (1999). TCR-mediated internalization of peptide-MHC complexes acquired by T cells. Science *286*, 952–954.

Ida, H., Nakashima, T., Kedersha, N.L., Yamasaki, S., Huang, M., Izumi, Y., Miyashita, T., Origuchi, T., Kawakami, A., Migita, K., et al. (2003). Granzyme B leakage-induced cell death: a new type of activation-induced natural killer cell death. Eur. J. Immunol. *33*, 3284–3292.

Idziorek, T., Estaquier, J., DeBels, F., and Ameisen, J.-C. (1995). YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J. Immunol. Methods *185*, 249–258.

Jameson, S.C. (2002). Maintaining the norm: T-cell homeostasis. Nat. Rev. Immunol. *2*, 547–556.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994a). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31–37.

Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994b). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science *265*, 528–530.

Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. (1996). Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. Annu. Rev. Immunol. *14*, 207–232.

Kagi, D., Odermatt, B., and Mak, T.W. (1999). Homeostatic regulation of $CD8^+$ T cells by perforin. Eur. J. Immunol. *29*, 3262–3272.

Kaiserman, D., Knaggs, S., Scarff, K.L., Gillard, A., Mirza, G., Cadman, M., McKeone, R., Denny, P., Cooley, J., Benarafa, C., et al. (2002). Comparison of human chromosome 6p25 with mouse chromosome 13 reveals a greatly expanded ov-serpin gene repertoire in the mouse. Genomics *79*, 349–362.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427–1429.

Kupfer, A., Singer, S.J., and Dennert, G. (1986). On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. J. Exp. Med. *163*, 489–498.

Lieberman, J. (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat. Rev. Immunol. *3*, 361–370.

Liu, N., Raja, S.M., Zazzeroni, F., Metkar, S.S., Shah, R., Zhang, M., Wang, Y., Bromme, D., Russin, W.A., Lee, J.C., et al. (2003). NF-kappa B protects from the lysosomal pathway of cell death. EMBO J. *22*, 5313–5322.

Liu, N., Phillips, T., Zhang, M., Wang, Y., Opferman, J.T., Shah, R., and Ashton-Rickardt, P.G. (2004). Serine protease inhibitor 2A is a protective factor for memory T cell development. Nat. Immunol. *5*, 919–926.

Lopez-Boado, Y.S., Espinola, M., Bahr, S., and Belaaouaj, A. (2004). Neutrophil serine proteinases cleave bacterial flagellin, abrogating its host response-inducing activity. J. Immunol. *172*, 509–515.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L.J., Robner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods *223*, 77–92.

Matloubian, M., Suresh, M., Glass, A., Galvan, M., Chow, K., Whitmire, J.K., Walsh, C.M., Clark, W.R., and Ahmed, R. (1999). A role for perforin in downregulating T-cell responses during chronic viral infection. J. Virol. *73*, 2527–2536.

McMichael, A. (1998). Preparing for HIV vaccines that induce cytotoxic T lymphocytes. Curr. Opin. Immunol. *10*, 379–381.

Medema, J.P., Schuurhuis, D.H., Rea, D., van Tongeren, J., de Jong, J., Bres, S.A., Laban, S., Toes, R.E.M., Toebes, M., Schumacher, T.N.M., et al. (2001a). Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells. J. Exp. Med. *194*, 657–667.

Medema, J.P., de Jong, J., Peltenburg, L.T.C., Verdegaal, E.M.E., Gorter, A., Bres, S.A., Franken, K.L.M.C., Hahne, M., Albar, J.P., Melief, C.J.M., and Offringa, R. (2001b). Blockade of the granzyme B/perforin pathway through over-expression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc. Natl. Acad. Sci. USA *98*, 11515–11520.

Medhurst, A., Harrison, D.C., Read, S.J., Campbell, C.A., Robbins, M.J., and Pangalos, M.N. (2000). The use of TaqMan RT-PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. J. Neurosci. Methods *98*, 9–20.

Menasche, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulffraat, N., Bianchi, D., Fischer, A., Le Deist, F., and de Saint Basile, G. (2000). Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat. Genet. *25*, 173–176.

Millard, P.J., Henkart, M.P., Reynolds, C.W., and Henkart, P.A. (1984). Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. J. Immunol. *132*, 3197–3204.

Odake, S., Kam, C.M., Narasimhan, L., Poe, M., Blake, J.T., Krahenbuhl, O., Tschopp, J., and Powers, J.C. (1991). Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins. Biochemistry *30*, 2217–2227.

Opferman, J.T., Ober, B.T., Narayanan, R., and Ashton-Rickardt, P.G. (2001). Suicide induced by cytolytic activity controls the differentiation of memory $CD8^+$ T lymphocytes. Int. Immunol. *13*, 411–419.

Perou, C.M., Justice, M.J., Pryor, R.J., and Kaplan, J. (1996a). Complementation of the beige mutation in cultured cells by episomally replicating murine yeast artificial chromosomes. Proc. Natl. Acad. Sci. USA *93*, 5905–5909.

Perou, C.M., Moore, K.J., Nagle, D.L., Misumi, D.J., Woolf, E.A., McGrail, S.H., Holmgren, L., Brody, T.H., Dussault, B.J., Jr., Monroe, C.A., et al. (1996b). Identification of the murine beige gene by YAC complementation and positional cloning. Nat. Genet. *13*, 303–308.

Peters, P.J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J.W., and Geuze, H.J. (1991). Cytotoxic T lymphocyte granules are secretory lysosomes, containing perforin and granzymes. J. Exp. Med. *173*, 1099–1109.

Phillips, T., Opferman, J.T., Shah, R., Liu, N., Froelich, C.J., and Ashton-Rickardt, P.G. (2004). A role for granzyme B inhibitor serine protease inhibitor 6 in $CD8^+$ memory cell homeostasis. J. Immunol. *173*, 3801–3809.

Pircher, H.-P., Moskophidis, D., Rohrer, U., Burki, K., Hengartner, H., and Zinkernagel, R.M. (1990). Viral escape by selection of cytotoxic T cell-resistant virus varients *in vivo*. Nature *346*, 624–632.

Podack, E.R., Young, J.D., and Cohn, Z.A. (1985). Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. Proc. Natl. Acad. Sci. USA *82*, 8629–8633.

Pope, C., Kim, S.K., Marzo, A., Masopust, D., Williams, K., Jiang, J., Shen, H., and Lefrancois, L. (2001). Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. J. Immunol. *166*, 3402–3409.

Revell, P.A., Grossman, W.J., Thomas, D.A., Cao, X., Behl, R., Ratner, J.A., Lu, Z.H., and Ley, T.J. (2005). Granzyme B and the downstream granzymes C and/or F are important for cytotoxic lymphocyte functions. J. Immunol. *174*, 2124–2131.

Russell, J.H., and Ley, T.J. (2002). Lymphocyte-mediated cytotoxicity. Annu. Rev. Immunol. *20*, 323–370.

Shi, L., Kraut, R.P., Aebersold, R., and Greenberg, A.H. (1992). A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J. Exp. Med. *175*, 553–566.

Silverman, G.A., Bird, P.I., Carrell, R.W., Church, F.C., Coughlin, P.B., Gettins, P.G.W., Irving, J.A., Lomas, D.A., Luke, C.J., Moyer, R.W., et al. (2001). The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. J. Biol. Chem. *276*, 33293–33296.

Spaner, D., Raju, K., Rudvanyi, L., Lin, Y., and Miller, R.G. (1998). A role for perforin in activation-induced cell death. J. Immunol. *160*, 2655–2664.

Spaner, D., Raju, K., Rabinovich, B., and Miller, R.G. (1999). A role for perforin in activation-induced T cell death in vivo: increased expansion of allogeneic perforin-deficient T cells in SCID mice. J. Immunol. *162*, 1192–1199.

Stinchcombe, J.C., Page, L.J., and Griffiths, G.M. (2000). Secretory lysosome biogenesis in cytotoxic T lymphocytes from normal and Chediak Higashi syndrome patients. Traffic *1*, 435–444.

Sun, J., Bird, C.H., Sutton, V., McDonald, L., Coughlin, P.B., De Jong, T.A., Trapani, J.A., and Bird, P.I. (1996). A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. J. Biol. Chem. *271*, 27802–27809.

Sun, J., Ooms, L., Bird, C.H., Sutton, V.R., Trapani, J.A., and Bird, P.I. (1997). A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9). J. Biol. Chem. *272*, 15434–15441.

Trambas, C.M., and Griffiths, G.M. (2003). Delivering the kiss of death. Nat. Immunol. *4*, 399–403.

Walden, P.R., and Eisen, H.N. (1990). Cognate peptides induce self-destruction of $CD8^+$ cytolytic T lymphocytes. Proc. Natl. Acad. Sci. USA *87*, 9015–9019.

Ward, D.M., Griffiths, G.M., Stinchcombe, J.C., and Kaplan, J. (2000). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. Traffic *1*, 816–822.

Ware, C.B., Siverts, L.A., Nelson, A.M., Morton, J.F., and Ladiges, W.C. (2003). Utility of a C57BL/6 ES line versus 129 ES lines for targeted mutations in mice. Transgenic Res. *12*, 743–746.

Wilson, S.M., Yip, R., Swing, D.A., O'Sullivan, T.N., Zhang, Y., Novak, E.K., Swank, R.T., Russell, L.B., Copeland, N.G., and Jenkins, N.A. (2000). A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. Proc. Natl. Acad. Sci. USA *97*, 7933–7938.

Zagury, D., Bernard, J., Thierness, N., Feldman, M., and Berke, G. (1975). Isolation and characterization of individual functionally reactive cytotoxic T lymphocytes. Conjugation, killing and recycling at the single cell level. Eur. J. Immunol. *5*, 812–818.

Zajac, A.J., Dye, J.M., and Quinn, D.G. (2003). Control of lymphocytic choriomeningitis virus infection in granzyme B deficient mice. Virology *305*, 1–9.