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A Role for the Granzyme B Inhibitor Serine Protease Inhibitor 6 in CD8+ Memory Cell Homeostasis

Tiphanie Phillips,* Joseph T. Opferman,† Ramila Shah,* Ni Liu,* Christopher J. Froelich,‡ and Philip G. Ashton-Rickardt*‡

Generation and maintenance of protective immunological memory is the goal of vaccination programs. It has recently become clear that CD8+ memory T cells are derived directly from CTLs. The mechanisms underlying this transformation and the subsequent survival of memory cells are not completely understood. However, some effector molecules required by CTLs to eliminate infected cells have also been shown to control the number of Ag-specific cells. We report that memory cells express high levels of serine protease inhibitor (Spi) 6, an inhibitor of the effector molecule granzyme B, and that Spi6 can protect T cells from granzyme B-mediated apoptosis. In mouse models, both elevated expression of Spi6 and the complete absence of granzyme B in CD8+ T cells led to an increase in memory cells after infection with lymphocytic choriomeningitis virus. This was not the result of increased levels of antilymphocytic choriomeningitis virus CD8+ T cells during the expansion or contraction phases, but rather transgenic Spi6 directly influenced the survival of CD8+ memory T cells. We propose that expression of protective molecules, like Spi6, serves to shield metabolically active CD8+ memory T cells from their own effector molecules. The Journal of Immunology, 2004, 173: 3801–3809.

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Abbreviations used in this paper: PCD, programmed cell death; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; PI, proteinase inhibitor; serpin, serine protease inhibitor; Spi, serine protease inhibitor.

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We examined the role of granzyme B and its inhibitor Spi6, on the development of CD8+ memory T cells after infection with lymphocytic choriomeningitis virus (LCMV). The expression of Spi6 was significantly up-regulated in CTLs and memory cells, which correlated with the expression of granzyme B. We observed increased levels of LCMV-specific memory cells in both Spi6 transgenic and granzyrne B cluster knockout (GnBKO) mice (32). However, surprisingly neither granzyrne B nor Spi6 affected the levels of effector cells generated at the peak of the response to LCMV or during the ensuing contraction phase. Further, wild-type and Spi6 transgenic memory cells experienced similar rates of cellular turnover. We conclude that Spi6 can influence the number of CD8+ memory T cells by prolonging survival and, therefore, Spi6 influences the homeostasis of memory cells by protecting them from granzyrne B.

Materials and Methods
Mice and virus
Fertilized eggs from C57BL/6 (B6) mice were microinjected with the Spi6 cDNA cloned into the human CD2 expression cassette (33) to generate two transgenic founders, which were identified by PCR with Spi6 primers: forward, 5′-GAA TTC CGG GCT GTA AGA GAC C-3′ and reverse, 5′-GGAG TAC TGA AGA GAG AAC TCT CCC-3′. Each founder was backcrossed to B6 mice to generate colonies of Spi6 Tg mice. The progeny from the backcrossing expressing Spi6 Tg cells were intercrossed to generate Spi6 Tg二代 mice. Homozygous status was verified by backcrossing to B6 mice and the identification of 20 consecutive Spi6 Tg offspring by PCR. Spi6 Tg二代 mice were crossed with P14 TCR transgenic mice (34), which had been backcrossed onto the B6 background, to generate P14 TCR+/Spi6 Tg二代 mice, which were further used to generate P14 TCR+/Spi6 Tg二代 mice by crossing with Spi6 Tg二代 mice. Spi6 Tg二代 mice were used for all experiments unless otherwise indicated. Additionally, the Spi6 cDNA was cloned into the 3x-Flag vector (Sigma-Aldrich, St. Louis, MO) and transfected into 293 T cells. Fibroblasts transfected with 3x-Flag-Spi6 cDNA was cloned into the 3x-Flag vector (Sigma-Aldrich, St. Louis, MO) and then cDNA was generated from unsorted B6 splenocytes) for each gene being analyzed. The sequence of the standard curve describes the efficiency of the real-time PCR. Only reactions that ran at >90% efficiency were included. An estimate of the amount of RNA in each experimental reaction was calculated using the equations of the standard curves. The data are reported as the ratio of the calculated amount of candidate RNA in a given sample by the calculated amount of the housekeeping control gene cyclophilin A in that same sample. Cyclophilin A was chosen as the housekeeping control because its expression level does not appear to be different between populations of T cells at different stages of activation (7).

Real-time PCR
RNA was extracted from purified cell populations using TRIzol Reagent (Invitrogen Life Technologies, San Diego, CA) and then cDNA was generated using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) (39). Primer and probe sequences for Spi6 (23): forward 5′-GCC ATC CAT CTT TGT AAG ATG C-3′, reverse 5′-TGG ACC CAA GAG AAC CAA CAT AGC-3′, probe 5′-TCC AAA AAT GTA TGT TAT TCT CCT GCG AGC ATC T-3′; and granzyrne B (32): forward 5′-CAAG ACC AGC TCT GTC CTT G-3′, reverse 5′-TGT CAG GTG GTT CCG AAC TTC CAC GTA AGA C TTC TGC CAC CA-3′; 1-Αά (40) (MHC class II): forward 5′-TGT TAG GAA TGA AGA CTG GAC CTT-3′, reverse 5′-CCA CGA GGC AGC TGA AGA TGT-3′; probe 5′-CAG ACA ACA GTA ATG CTA GGG ATG ATC CCA-3′; and cyclophilin A (39): forward 5′-CCA TAA CAC CAT TCC TTC TGT ACG-3′, reverse 5′-AGC GAT TAG AGG ACA TTA CTA GCA-3′. The probe 5′-CAG GGA GTA TGC CCA-3′ were designed using Primer Express software (PE Applied Biosystems, Foster City, CA). The unique specificity of each set was verified by checking the sequences against GenBank database. Real-time PCR were conducted using TaqMan Universal PCR Master Mix (PE Applied Biosystems) and run on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Data were captured and analyzed using Sequence Detection software (PE Applied Biosystems).

FACS analysis
All fluorochrome-conjugated mAbs were purchased from BD Pharmingen (San Diego, CA). H-2Db tetramers used to detect Ag-specific cells in the peripheral blood and spleen were generated as previously described (35). CD8+ T cell populations used for real-time PCR were sorted to >97% purity using a MoFlo (DAKO Cytomation, Carpinteria, CA). Briefly, single cell suspensions were prepared, by depletion of erythrocytes with ammonium chloride and purification with Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada), from the pooled spleens (5–10 mice) of naive or LCMV Armstrong infected B6 mice either 8 days (effector) or 50–100 days (memory) after infection. Splenocytes from naive mice were FACs purified directly after staining with anti-CD8α allophycocyanin and anti-CD44 PE mAbs. Splenocytes from effector and memory mice were prepared using Lympholyte-M (Cedarlane Laboratories) and the RNA extracted using TRIzol Reagent (Invitrogen Life Technologies). RNA was enriched for mRNA using MicroFastTrack mRNA isolation kit (Invitrogen Life Technologies). Equal amounts of mRNA were run on a 1.5% gel, transferred to a Hybond-N+ membrane (Amersham Biosciences) and the membrane hybridized overnight with a 32P-labeled Spi6 cDNA. The final wash was in 0.1× SSC with 0.5% SDS at 65°C and the membrane was exposed to film for 6 h. The membrane was then stripped and reprobed with a 32P-labeled GAPDH cDNA. The final wash was in 0.5× SSC with 0.5% SDS at 65°C and the membrane was exposed to film for 4 h.

Apoptosis assays
Jurkat cells (human thymoma) were cotransfected with 5 μg of either the Spi6 cDNA cloned into the CD2 expression cassette (33) or empty vector and PGK-Neo (5 μg) by electroporation (280 V, 975 μF). Transfectants were selected and cloned by growing in G418 (Invitrogen Life Technologies) over a 3-wk period. Clones were treated for 2 h with human perforin (10 μg/ml) or human granzyme B (2 μg/ml) (41, 42), or cultured overnight with anti-human Fas IPO-4 mAb (0.12 μg/ml) (43) or subjected to gamma-irradiation (4456 rad) and apoptosis measured after 20 h. The early onset of apoptosis was detected in non-necrotic cells (propidium iodide-negative) by staining with YOPPO-1, according to the manufacturer’s instructions (Molecular Probes, Eugene, OR) (44).

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Adoptive transfers

Naive CD8$^+$ T cells were purified (>90% pure) from the spleens of P14 or P14 × Spi6 mice (Thy1.2$^+$) by positively sorting with anti-CD8 magnetic beads (Miltenyi Biotec), then adoptively transferred (10$^6$) by i.v. injection into Thy1.1$^+$ B6 mice. Recipients were rested for 2 days and then infected with LCMV. Thy1.2$^+$ donor P14 or P14 × Spi6 cells were followed in the peripheral blood of recipients by staining with anti-Thy1.2 mAbs.

Brdu incorporation

The level of memory cell turnover was determined by incorporation of Brdu. LCMV-immune Thy1.1$^+$ mice that had received either P14 or P14 × Spi6 cells (Thy1.2$^+$) were given Brdu (Sigma-Aldrich) for 1 wk in their drinking water (0.8 mg/ml) and then analyzed. Briefly, $10^6$ splenocytes were surface stained using anti-Thy1.2 PE mAbs and then fixed using Cytofix/Cytoperm solution (BD Pharmingen). Fixed cells were washed once in Perm/Wash solution (BD Pharmingen) both before and after incubation with 100 U DNase I (Sigma-Aldrich) for 2 h at 37°C. Cells were then stained with anti-Brdu FITC (BD Pharmingen) or isotype control (BD Pharmingen), washed in Perm/Wash solution, and analyzed by FACS.

Statistical analyses

All p values were determined using t tests. Statistical analyses shown in first figure were performed using all values obtained for all isolates.

Results

Spi6 is up-regulated in effector and memory cells

Before beginning to address a physiological role for Spi6 in the development of memory cells, we first examined the expression pattern of Spi6 in CD8$^+$ T cells after infection with LCMV. Anti-LCMV cells were purified (>97% pure) 8 days (effectors) or more than 50 days (memory cells) after infection by staining with anti-CD8a mAbs and H-2D$^b$ tetramers loaded with the dominant NP396, GP33, and GP276 LCMV peptides (Fig. 1A) (37). Naive cells (CD8$^+$CD44low) were purified (99% pure) from the spleens of uninfected B6 mice (Fig. 1A).

Real-time PCR using primers and probes specific for Spi6 and the housekeeping gene cyclophilin A was performed on cDNA generated from RNA isolated from the purified cell populations (39). Two separate isolates each of naive, effector, and memory cells were assessed for Spi6 expression. One representative value for each isolate is expressed in Fig. 1B as the ratio of Spi6 to cyclophilin. Naive cells did not express high levels of Spi6 (an average ratio of 0.6), but 8 days after LCMV infection, Ag-specific effectors had significantly up-regulated Spi6 expression (an average ratio of 11.9, p < 0.001 compared with naive cells). Therefore, separate isolates of effector cells were found to express Spi6 28.6-fold and 11-fold higher than naive cells. A third independent isolate of effector cells was also assessed for Spi6 expression and found to be 53-fold higher than naive cells (an Spi6 to cyclophilin ratio of 31.7, data not shown). This up-regulation is consistent with the original identification of Spi6 in a CTL cell line (23). In memory cells, the expression of Spi6 fell 3.4-fold from the level detected in effectors (an average ratio of 3.5, p < 0.05 compared with effectors), but remained, on average, 5.8-fold higher than naive cells (p < 0.001).

The expression pattern of Spi6 in CD8$^+$ T cells correlated with that of granzyme B (Fig. 1C), the granule protease inhibited by Spi6 (23, 31). Granzyme B expression was hugely up-regulated in effector cells (>200-fold higher than naive cells, p < 0.001). The expression of granzyme B, although 24-fold lower than effectors (p < 0.001), was retained in memory cells, which expressed ~9-fold higher levels of granzyme B than naive cells (p < 0.01). This result is consistent with previous reports of granzyme B transcript and protein expression in populations of CD8$^+$ T cells (7, 45, 46). The level of MHC class II expression in all populations of purified cells was negligible (Fig. 1D), indicating that no contaminating APCs were present. Real-time PCR analysis, therefore, indicated that Spi6 is coexpressed with granzyme B in anti-LCMV effectors, and expression of both is retained in the resulting memory cells. This suggests a role for Spi6 in protecting cytolytic CD8$^+$ T cells from granzyme B. Verification of the up-regulation of Spi6 protein was precluded by the lack of an available Ab.

![Figure 1](https://www.jimmunol.org/abstracts/3803/fig1.png)
Spi6 protects T cells specifically from granzyme B-mediated apoptosis

Although it has previously been shown that Spi6 can protect mature DCs and naturally occurring tumors from the granule exocytosis pathway (30, 31), we tested directly whether, similar to its human homologue P19, Spi6 can protect T cells from granzyme B. Cloned transfectants of Jurkat cells that expressed Spi6 under the control of a human CD2 expression cassette (33) (0.45 and 0.31 ng per ng of rRNA compared with <0.001 ng of Spi6 mRNA per nanogram of rRNA in untransfected controls) were used to test the ability of Spi6 to protect T cells from granzyme B-induced PCD. Granzyme B, in the presence of sublytic concentrations of perforin, induced significantly less ($p < 0.05$) apoptosis in Spi6 transfectants compared with controls (Fig. 2A) (41). This protection was specific to granzyme B, because the percentage of apoptosis initiated by either ligation of the Fas death receptor (43) or gamma-irradiation was no different between Spi6 transfectants and controls (Fig. 2B). Therefore, as with the human granzyme B inhibitor P19 (42), expression of Spi6 can protect T cells specifically from death initiated by granzyme B delivered by perforin.

Generation and characterization of Spi6 transgenic mice

To study the role of Spi6 in CD8+ T cell biology in vivo, we generated transgenic mice expressing the 1.1 kb Spi6 cDNA under the control of the human CD2 promoter (33). Two founder mice were backcrossed to B6 mice to generate colonies of mice expressing heterozygous transgenic Spi6, and the offspring of these mice intercrossed to generate homozygous transgenic Spi6 mice (referred to hereafter as Spi6 mice).

The endogenous level of Spi6 in CTLs was significantly higher than that of naive CD8+ T cells (Fig. 1B). Therefore, we needed to confirm that transgene-driven expression of Spi6 in effectors increased the total level of Spi6 mRNA over that of the endogenous level. We crossed Spi6 mice to transgenic mice expressing the P14 TCR, which recognizes the GP33 peptide from LCMV in the context of H-2Db (34). Splenocytes from P14 and P14 × Spi6 mice were cultured with GP33 peptide and IL-2 for 3 days, at which time >90% of the cells have become activated P14 cells (data not shown). Northern blot analysis of poly(A)+ RNA confirmed the expression of the endogenous Spi6 mRNA (2.4 kb) in all P14 CTLs (Fig. 3A) (23). Effectors from P14 × Spi6 mice, however, expressed high levels of a smaller transgenic Spi6 transcript (~2.1 kb). The transgenic transcript lacks the 3’ untranslated region of the endogenous transcript and terminates at a CD2-specific sequence (33). Overall, therefore, the level of Spi6 was significantly higher in CTLs from Spi6 mice.

FIGURE 2. Spi6 protects T cells specifically from granzyme B-mediated apoptosis. A, Jurkat clones transfected with the Spi6 cDNA or CD2 expression cassette vector (V) were incubated in the presence of sublytic concentrations of perforin with (■) or without (□) granzyme B, or (□) in medium alone (○). Apoptosis was significantly reduced in the Jurkat clones transfected with Spi6, compared with Jurkat clones transfected with vector alone. B, Transfected Jurkat clones were cultured in the presence of medium alone (○). Ab against Fas (anti-Fas), or exposed to gamma-irradiation (γ-IR). Apoptosis initiated by these stimuli did not differ between Jurkat clones transfected with Spi6 compared with controls, indicating that Spi6 protects specifically from granzyme B-mediated apoptosis. Histograms are the mean ± SEM from three determinations. *, $p < 0.05$.

FIGURE 3. The expression of Spi6 in B6 and Spi6 mice. A. Poly(A)+ RNA for Northern blots was isolated from CTLs generated from P14 mice and mice heterozygous (P14 × Spi6 (het)) or homozygous (P14 × Spi6 (homo)) for transgenic Spi6 expression. Hybridization with a 32P-labeled Spi6 cDNA probe identified the endogenous (2.4 kb) and transgenic (~2.1 kb) Spi6 transcripts. Blots were stripped and reprobed with a 32P-labeled GAPDH cDNA to reveal equal loading of mRNA. B. Cells were isolated from two to four B6 and Spi6 mice (C), pooled, and the expression level of Spi6 determined by real-time PCR. Histograms are the mean of three determinations.
The CD2 expression cassette also drives the expression of genes in cells other than CD8⁺ T cells (33). Analysis of Spi6 expression was performed by real-time PCR on various hematopoietic cell populations. Endogenous expression of Spi6 in B6 mice was found in all cell types tested, with NK cells expressing the highest levels (Fig. 3B). The transgene increased expression of Spi6 at least 40-fold in all cell types, with the highest expression levels in thymocytes and other lymphocytes (Fig. 3C).

Importantly, increased expression of Spi6 in transgenic mice did not alter thymic development because the numbers of CD4 single-positive, CD8 single-positive, and CD4 CD8 double-positive thymocytes were no different in Spi6 and B6 mice (Table I). Additionally, the number of B cells and T cells in the spleen and lymph nodes of Spi6 mice were no different from the numbers in B6 mice (Table I). Although, on average, the percentage of T cells that were memory-phenotype (CD44high) in Spi6 mice was slightly higher than B6 mice (CD44high of CD4⁺ cells in lymph node: 13.9 ± 1.4% vs 10.7 ± 0.9% and in spleen: 23.1 ± 1.1% vs 17.5 ± 1.7%; CD44high of CD8⁺ cells in lymph node: 19.3 ± 1.0% vs 17 ± 1.6%, and in spleen: 24.9 ± 0.6% vs 22.9 ± 2.0%), this difference was not significant (p > 0.05, data not shown).

Enhanced development of memory cells in Spi6 mice after LCMV infection

Spi6 expression was highly up-regulated in effectors and the resulting memory cells (Fig. 1B). To assess whether Spi6 plays a role in the development of CD8⁺ T cell memory, we infected Spi6 mice with LCMV and quantitated the number of memory cells that developed. A hallmark of memory cells is the ability to respond immediately to Ag rechallenge (8). Therefore, 180 or more days after LCMV infection, we measured the number of CD8⁺ memory T cells that produced IFN-γ after ex vivo stimulation with Ag peptide (37). Infection of Spi6 mice resulted in significantly more memory cells compared with B6 mice, whether represented as a percentage of splenocytes (p < 0.05 or 0.01, Fig. 4A) or by absolute cell number (p < 0.05, Fig. 4B). Specifically, compared with B6 mice memory cell numbers in Spi6 mice were 2.9-fold higher for cells recognizing the GP33 peptide, 2.1-fold higher for cells recognizing the NP396 peptide and 2.8-fold higher for cells recognizing the GP276 peptide.

If Spi6 increased the amount of memory cells through the inhibition of granzyme B-mediated PCD, the absence of granzyme B would be predicted to have a similar effect on memory cell development. Therefore, we determined the number of memory cells generated after LCMV infection of GrnBKO mice (32). The presence of granzyme B is not essential for resolution of an LCMV infection, so there is no persisting Ag and the kinetics of CD8⁺ T cell expansion and contraction can be followed in GrnBKO mice (47). Similar to Spi6 mice, GrnBKO mice also had elevated numbers of memory cells compared with B6 mice (Fig. 4, A and B). We detected 2.2-fold more memory cells recognizing the GP33 peptide (p < 0.05), 1.8-fold more memory cells recognizing the NP396 peptide and 2.3-fold more memory cells recognizing the GP276 peptide (p < 0.05) in GrnBKO mice.

The increase in the number of memory cells in Spi6 and GrnBKO mice was not the result of increased clonal burst size. Eight days after infection, the peak level of anti-GP33 CD8⁺ cells in Spi6 (7.3 ± 0.5%), B6 (7.1 ± 0.4%) and GrnBKO mice (8.2 ± 0.5%) was no different (Fig. 4C). In addition, the increase in memory cell numbers was also not attributable to an effect on the contraction phase, as 15 days postinfection the percentages of anti-GP33 CD8⁺ cells in Spi6 (5.0 ± 0.4%), B6 (5.0 ± 0.4%) and GrnBKO mice (5.5 ± 0.3%) were also equivalent (Fig. 4C). Interestingly, at 30 days postinfection the percentage of anti-GP33 CD8⁺ cells in Spi6 (4.4 ± 0.4%) and GrnBKO mice (5.0 ± 0.4%) was higher than B6 mice (3.5 ± 0.4%), although this was not yet significant (p > 0.05, Fig. 4C). Together, these data support the hypothesis that granzyme B can influence the number of anti-LCMV memory cells, not by altering the expansion or contraction phases, but by acting during the memory phase.

![Figure 4](http://www.jimmunol.org/)

**Figure 4.** Enhanced memory cell development in Spi6 mice. Spi6 (□), B6 (■), and GrnBKO (□) mice were infected with LCMV, and memory cells detected in the spleen >180 days later by measuring ex vivo IFN-γ production. A, Spi6 and GrnBKO mice had significantly higher percentages of memory cells than B6 mice. B, The absolute number of memory cells generated was higher in Spi6 mice than B6 mice (GP33: 2.9 times more; NP396: 2.1 times more; GP276: 2.8 times more). GrnBKO mice also generated more memory cells than B6 mice (GP33: 2.2 times more; NP396: 1.8 times more; GP276: 2.3 times more). C, The percentages of Ag-specific cells in the blood of Spi6, B6, and GrnBKO mice, detected using H-2D^b tetramers loaded with GP33 peptide, were not significantly different 8, 15, or 30 days postinfection. Histograms are the mean ± SEM from combined experiments (n = 7–9 mice per group). *, p < 0.05; **, p < 0.01, when compared with B6 mice.
Cell-autonomous Spi6 expression increases the frequency of memory cells

Spi6 mice have elevated expression levels of Spi6 in several types of hemopoietic cells (Fig. 3C). Therefore, the increase in memory cell numbers detected in Spi6 mice could be the result of enhanced priming (48) by Spi6 transgenic DCs (30). To examine the role of Spi6 in CD8<sup>+</sup> T cells during the development of memory, we examined the anti-LCMV response of P14 × Spi6 cells after adoptive transfer, a system that has been well characterized (7, 49, 50). Naive CD8<sup>+</sup> T cells were purified from P14 and P14 × Spi6 mice (both Thy1.2<sup>+</sup>) and then adoptively transferred (10<sup>5</sup>) into Thy1.1<sup>+</sup> congenic B6 recipients. After 2 days, recipients were infected with LCMV and the levels of P14 memory cells determined after at least 50 days by ex vivo IFN-γ production.

Gating on Thy1.2<sup>+</sup> or Thy1.2<sup>+</sup> cells during FACS analysis allowed us to detect the endogenous (Thy1.2<sup>+</sup>) and donor (Thy1.2<sup>+</sup>) memory cells (CD8<sup>+</sup> IFN-γ<sup>+</sup>) in recipients (Fig. 5A). We observed elevated levels of P14 × Spi6 memory cells in recipients compared with P14 memory cell levels, an average of 1.5 ± 0.16% vs 0.61 ± 0.15% (p < 0.01, Fig. 5B). When the data from several experiments were pooled and normalized there were twice as many P14 × Spi6 memory cells as P14 memory cells (2.0 ± 0.2% vs 1.1 ± 0.3%, p < 0.01, Fig. 5C). The same was true for the actual percentage (not normalized) of P14 × Spi6 memory cells compared with P14 memory cells (0.85 ± 0.13% vs 0.48 ± 0.12%, p < 0.05, data not shown). Normalized memory cell percentages were calculated proportionally by considering the percentage of Thy1.2<sup>+</sup> cells in the peripheral blood of recipients at the peak of the response 7 days postinfection as 100%. Importantly, the percentages of endogenous (Thy1.2<sup>+</sup>) memory cells generated in recipients receiving P14 cells did not differ from those recipients receiving P14 × Spi6 cells (Fig. 5D).

As in the experiments using intact animals (Fig. 4C), there was no difference in the level of P14 and P14 × Spi6 cells at the peak of the response on day 7 (44.7 ± 1.7% vs 41.3 ± 1.8%, Fig. 5E) or during the contraction phase, measured on day 14 (15.4 ± 1.2% vs 15.0 ± 1.1%, Fig. 5E). Interestingly, 28 days postinfection more P14 × Spi6 cells could be detected in recipients than P14 cells (7.7 ± 0.9% vs 5.7 ± 1.2%, Fig. 5E). However, unlike the difference detected after at least 50 days (Fig. 5C), the difference at this early time point was not yet significant (p > 0.05). Transgenic Spi6 expression, therefore, led to an increased frequency of anti-LCMV memory cells by directly protecting CD8<sup>+</sup> T cells, not during the expansion or contraction phases, but rather during the memory phase.

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**FIGURE 5.** Expression of Spi6 in CD8<sup>+</sup> T cells increases the level of memory cells. Naive CD8<sup>+</sup> T cells from P14 and P14 × Spi6 mice, both Thy1.2<sup>+</sup>, were purified using magnetic beads and adoptively transferred to Thy1.1<sup>+</sup> congenic recipients. Recipients were infected with LCMV and more than 50 days later the percentage of memory cells in recipient spleens determined by measuring ex vivo IFN-γ production. A. FACS scans from representative recipients given P14 or P14 × Spi6 cells. Total live cells did not stain with the isotype control (IC) when stimulated with GP33 peptide, nor did they stain for IFN-γ production in the absence of stimulation (No peptide). Donor and recipient memory cells were CD8<sup>+</sup> IFN-γ<sup>+</sup> and Thy1.2<sup>+</sup> or Thy1.2<sup>+</sup>, respectively. Stimulation with GP33 revealed that virtually all (91–98%) of the Thy1.2<sup>+</sup> cells were functional CD8<sup>+</sup> IFN-γ<sup>+</sup> memory cells. B. The percentages of P14 memory cells (●, mean: 0.61 ± 0.15%) or P14 × Spi6 memory cells (○, mean: 1.5 ± 0.16%) from individual mice are indicated for one experiment. C. Percentages of memory cells from four experiments were normalized to the percentage of P14 or P14 × Spi6 cells (Thy1.2<sup>+</sup>) in the blood 7 days after infection with LCMV. The normalized percentage of P14 × Spi6 memory cells was significantly higher than P14 memory cells. D. The percentage of resident memory cells (Thy1.2<sup>+</sup>) was no different between recipients receiving P14 cells or P14 × Spi6 cells (p > 0.05). E. There was no significant difference (p > 0.05) in the level of Thy1.2<sup>+</sup> donor P14 or P14 × Spi6 cells in the blood 7, 14, or 28 days after infection of recipients with LCMV (p > 0.05). Histograms are the mean ± SEM from four pooled experiments (n = 12–14 mice per group). **, p < 0.01.
Formally, the increased frequency of Spi6 transgenic memory cells could be due to either enhanced proliferation of memory cells containing transgenic Spi6 or to increased long-term survival. To help distinguish between these possibilities, LCMV-immune Thy1.1+ mice that had received P14 or P14 × Spi6 cells were given BrdU in their drinking water and 1 wk later the turnover of memory cells in the spleens of these animals determined by BrdU incorporation. The frequency of P14 and P14 × Spi6 memory cells that had divided (18 ± 0.5% vs 18.1 ± 0.4%) was the same, indicating that memory cells containing transgenic Spi6 appear to proliferate similarly to wild-type memory cells. This finding supports the idea that transgenic Spi6 increases the long-term survival of memory cells rather than increasing proliferation. In fact, FACS-purified Spi6 transgenic memory cells from LCMV-immune mice expressed levels of Spi6 (3.1 ng of Spi6 mRNA per nanogram of rRNA) higher than those capable of protection of Jurkat clones from granzyme B in vitro (0.45 and 0.31 ng of Spi6 mRNA per nanogram of rRNA, Fig. 2). In conclusion, transgenic Spi6 expression can improve the survival of memory cells by protecting them in a cell-autonomous fashion, supporting the idea that granzyme B contributes to CD8+ memory T cell homeostasis.

Discussion

The level of CD8+ memory T cells is determined by events in the expansion, contraction and memory phases of the immune response (1). We report that CD8+ memory T cells express elevated levels of Spi6, and that inhibition of granzyme B (by deficiency or expression of transgenic Spi6) increases the number of CD8+ memory T cells. We propose that Spi6 improves the survival of metabolically active CD8+ memory T cells by protecting them from their own granzyme B effector molecules.

Previous studies have indicated that Spi6 can inhibit granzyme B and, importantly, protects cells from the granule exocytosis pathway of PCD (23, 30, 31). We extend these studies and demonstrate that Spi6 can protect T cells from granzine B-mediated PCD (Fig. 2). We interpret the increase in anti-LCMV memory cells in Spi6 and GrnBKO mice as evidence that granzyme B can act as a negative regulator in CD8+ memory T cell homeostasis through the induction of PCD (Figs. 4 and 5). An earlier study reported that GrnBKO mice have wild-type levels of anti-LCMV memory cells (47). This discrepancy could be the result of the differences in the method of memory cell detection (ELISPOT vs intracellular cytokine staining) or that the earlier study was conducted on GrnBKO mice of the B6 × 129 background and in our study we used B6 GrnBKO mice. Other effector molecules, perforin and IFN-γ, have previously been shown to influence Ag-specific cells during the expansion and contraction phases (16, 18–21), so it would appear that effector molecules can regulate the levels of CD8+ T cells throughout the entire course of an immune response.

The enhanced memory cell phenotype we report in Spi6 mice (2- to 3-fold higher than B6 mice, Fig. 4) is about the same magnitude observed in other transgenic systems with higher memory cell levels, after over-expression of calcium/calmodulin kinase II and the Bcl-6 transcriptional repressor (51, 52). Additionally, transgene driven expression of Bcl-2 and Bcl-xL, two well known anti-apoptotic proteins, did not lead to increases in memory cell numbers after infection, even though over-expression of these proteins protected effector cells from apoptosis in vitro (49). So, simply over-expressing an anti-apoptotic protein in CD8+ T cells does not account for the development of increased memory cell levels in Spi6 mice.

Immunological memory not only requires the initial differentiation of CD8+ memory cells, but also their long-term maintenance (1). The impact of some molecules, such as the IL-15 cytokine, on memory cells is, in fact, a result of their action well past the contraction phase of CD8+ memory T cell development (53). Consistent with previous reports (47), we did not find persistently elevated levels of effectors or an altered contraction phase after infection of GrnBKO mice with LCMV, a phenotype mimicked in Spi6 mice (Fig. 4C). Therefore, the impact of inhibition of granzyme B seems to be specific to the memory phase of the response to LCMV and did not lead to autoimmunity or immunopathology (Figs. 4 and 5, data not shown). This is in stark contrast to the phenotype of perforin deficiency, which results in elevated levels of effectors, delayed contraction and pathology (16, 18). However, perforin-deficient mice have a defect in pathogen clearance that leads to persistence of Ag, which drives dysregulation of effectors in these animals. Neither GrnBKO (47) nor Spi6 mice have a defect in viral clearance (data not shown). Therefore, Ag did not persist to drive effector cell dysregulation. Under these conditions of normal viral clearance, true memory cell development could be assessed in the GrnBKO and Spi6 mice, unlike in perforin-deficient mice. However, the massive increases in the numbers of activated CD8+ T cells in perforin-deficient mice and humans clearly demonstrate a role for the granule exocytosis pathway of PCD in the control of activated CD8+ T cells. Clearly, granule proteases other than granzyme B can limit clonal burst size and eliminate CD8+ T cells after infection, or are able to compensate for granzyme B in its absence.

The finding that transgenic Spi6 expression in memory cells reciprocated the phenotype of granzyme B deficiency is consistent with Spi6 being an endogenous inhibitor of granzyme B in CD8+ T cells (Fig. 2). Further, that the Spi6 and GrnBKO mouse memory phenotypes are similar suggests that the amount of Spi6 expressed in the Spi6 transgenic memory cells was sufficient to block all the granzyme B capable of initiating apoptosis. This explains why the massive up-regulation of Spi6 did not increase memory cell numbers higher than 3-fold, as transgenic Spi6 can only inhibit all the granzyme B.

Despite increasing the number of memory cells, transgenic Spi6 did not affect the level of CTLs (Figs. 4C and 5E). Importantly, this is not to say that effectors are not protected from granzyme B by Spi6. Wild-type effectors may simply up-regulate Spi6 enough endogenously to protect themselves as much as they can from the granzyme B capable of inducing effector cell apoptosis during the expansion and contraction phases (Fig. 1B). In this case, further expressing Spi6 in the effectors did not have an additional protective effect. However, once the endogenous level decreases somewhat to the level in memory cells, the additional Spi6 in the Spi6 mice protected the memory cells at a level they do not ordinarily achieve endogenously. It will be interesting to investigate whether granzyme B is capable of inducing Ag-specific cell death during the expansion and contraction phases in a Spi6-deficient mouse model.

One of the salient qualities of memory cells is the ability to respond quickly to Ag, mediated, in part, because they have pre-formed granules containing toxins such as perforin and granzyme B (6, 45). It has recently been reported that this immediate response is the result of a specialized G0/G1 cell cycle state predisposing memory cells to rapid division upon stimulation (12). At any given time, a certain percentage of the memory cell population is in cell cycle (10), and the ability of memory cells to cycle is absolutely critical to an anamnestic response (11). Long-lived memory cells, therefore, exist in a metabolically active state over a long period of time in the presence of cytotoxins, and we would suggest this makes them susceptible to death induced by those toxins. PI9, the human homologue of Spi6, can protect both NK
cells and CTLs from PCD by inhibiting "misdirected" granzyme B that has leaked into the cytoplasm (27, 28). Because memory cells retain expression of granzyme B, we suggest that coexpression of Sp6 protects these cells in a similar fashion and improves the long-term survival of memory cells. We propose that granzyme B and its inhibitor, Sp6, can function in an antagonistic fashion, providing a pro- and anti-apoptotic balance that contributes to the homeostasis of metabolically active CD8+ memory T cells. Activated CD8+ T cells appear to selectively leak granzyme B from their granules (and not granzyme A or perforin) (54). This could explain why effector cells up-regulate Sp6. It has yet to be determined whether this selective leakage occurs in true memory cells, but it is possible that memory cells also leak granzyme B from their granules and are susceptible to death initiated by cytoplasmic granzyme B if it is not inhibited.

The data reported support the idea that granzyme B is capable of initiating apoptosis in Ag-specific cells after the contraction phase as the level of endogenous Sp6 decreases. Transgenic Sp6 protected Ag-specific cells as they transitioned into memory cells, so we were able to detect a small increase in Ag-specific cell number from day 15 to day 50, when the difference had become large enough to be statistically significant. Because they retain granzyme B expression, memory cells appear to be susceptible to granzyme B-initiated apoptosis. However, we would not suggest that granzyme B is the only initiator of apoptosis regulating the number of memory cells. It is clear from the plateau in the difference in number of Sp6 and wild-type memory cells that is reached (day 50 and 180 frequencies are similar, Figs. 4A and 5B) that the memory cells containing transgenic Sp6 do not continue to expand without control.

Some virulent strains of LCMV have been shown to persist in vivo instead of being cleared (55). Under conditions of persistent Ag exposure, the CD8+ T cell response can clonally exhaust. During clonal exhaustion the Ag-specific cells that cannot clear the pathogen undergo progressive dysfunction and are eventually completely eliminated, possibly to avoid the induction of autoimmunity during prolonged activation (56). The mechanism(s) responsible for clonal exhaustion are not clear. Although other molecules that can influence the numbers of activated Ag-specific CD8+ T cells, such as perforin, IFN-γ and Fas, have been implicated in clonal exhaustion (57, 58), to our knowledge there is no evidence to suggest that granzyme B plays a role in this process. Therefore, we would not predict that Sp6 mice would have an alteration compared with wild-type mice in their response to infection with a persistent virus. Further, in a clonal exhaustion situation, a true memory phase is never achieved. As we report only an influence of Sp6 on memory cells, this is further reason to speculate that the response of Sp6 mice to persistent Ag would not differ from wild-type.

The human homologue of Sp6, P19, not only inhibits granzyme B, but also other proteases such as elastase, and even the cytotoxic protease caspase-1 (59, 60). Therefore, it is possible that, in addition to granzyme B, Sp6 protects CD8+ T cells from PCD through inhibition of other executioner proteases. The generation of Sp6-deficient mice will allow for this possibility to be tested. Mice express a greater number of granzymes (61) and serpins (29) than humans, and so careful analysis of the roles of these proteins in mouse models will provide a better understanding of the development of protective CD8+ T cell immunity.

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References


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