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Differential Survival of Cytotoxic T Cells and Memory Cell Precursors

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It is widely assumed that the development of memory CD8 T cells requires the escape of CTLs from programmed cell death. We show in this study that although serine protease inhibitor 6 (Spi6) is required to protect clonal bursts of CTLs from granzyme B-induced programmed cell death, it is not required for the development of memory cells. This conclusion is reached because memory cell precursors down-regulate both Spi6 and granzyme B, unlike CTLs, and they do not require Spi6 for survival. These findings suggest that memory CD8 T cells are derived from progenitors that are refractory to self-inflicted damage, rather than derived from fully differentiated CTLs. The Journal of Immunology, 2007, 178: 3483–3491.

Recognition of peptide Ag, presented by self-MHC (pMHC), by the TCR triggers the clonal expansion and differentiation of naive CD8 T cells into CTLs (1). CTLs kill target cells either by exocytosis of cytolytic granules or the engagement of Fas by Fas ligand (2). Exocytosis of perforin (3, 4) facilitates the entry of serine proteases called granzymes, which trigger apoptosis in target cells. Granzyme B (GrB) is required for the induction of target cell death (5, 6), through the activation of apoptotic pathways triggered by the cleavage of caspases and the induction of nuclear DNA fragmentation (7).

The clonal expansion (or clonal burst) of naive CD8 T cells into CTLs is followed by the contraction phase of the immune response, during which 90–95% of effectors are eliminated through programmed cell death (PCD) (1). Those cells that survive the contraction phase subsequently develop into memory CD8 T cells (8, 9). The memory phase can extend for the lifetime of the host (1), providing immunity as the result of both an increased precursor frequency of Ag-specific cells and improved function compared with naive cells (1). Memory cell development in which size of the clonal burst is flexible and the degree of contraction fixed, allows the generation of memory CD8 T cells proportional to the primary CTL response, while leaving space for CD8 T cell responses against new infections. Thus, 5–10% of the CTL pool survives to differentiate into memory CD8 T cells, and so clonal burst size determines the size of the memory pool (10–15).

Studies from our laboratory (8) and other studies (9, 16) indicate that memory CD8 T cells are derived from the CTL-progenitor lineage. Therefore, to understand how memory precursors escape PCD, emphasis has been placed on understanding the pathways that control CTL survival. Effector molecules induce the PCD of CTLs and thereby play a role in determining both clonal burst size and the severity of the contraction phase. The clonal burst size of CTLs is massively increased in perforin knockout (KO) CTLs (17–19) and IFN-γ KO CD8 T cells fail to undergo a contraction phase (20, 21). These studies strongly suggest that PCD triggered by perforin determines clonal burst size and PCD triggered by IFN-γ controls the severity of the contraction phase. The expression of IL-7R (CD127) identifies a small percentage of CTLs, which have an increased likelihood of differentiating into memory CD8 T cells (22, 23). These IL-7R^high, memory precursors up-regulate protective factors, which allows them to escape PCD and differentiate into memory cells (24). However, it remains to be determined whether the biochemical pathways that control the survival of CTLs are the same as those that control the survival of memory cell precursors and the subsequent differentiation of memory CD8 T cells.

We have recently shown (25), that the clonal burst size of CTLs is determined by an endogenous inhibitor of Grb called serine protease inhibitor 6 (Spi6) (26). Spi6 protected CTLs from PCD by inactivating Grb that leaks from cytotoxic granules in the cytoplasm through the formation of complexes characteristic of a serpin: protease interaction. The survival of Spi6 KO CTLs specific for lymphocytic choriomeningitis virus (LCMV) or *Listeria monocytogenes* (LM) was drastically impaired due to increased Grb-mediated PCD. We made use of Spi6 KO mice to determine whether Spi6 in turn was required to promote the survival of memory cell precursors and hence development of memory CD8 T cells.

Infection with LCMV revealed a critical requirement for Spi6 in determining the clonal burst size of both primary and secondary CD8 T cell responses to *L. monocytogenes* and LCMV. To our surprise, despite a severely diminished clonal burst, the number of memory CD8 T cells was unaffected in Spi6 KO mice because although Spi6 was required for the survival of the majority CTLs, it was not needed by the subset of IL-7R^high memory cell precursors. The independence of memory cell precursors from Spi6 protection from Grb was because they constituted a lineage in which...
GrB and Spi6 were down-regulated. The resulting model of CD8 memory T cell development is one in which independence from self-inflicted damage defines the population of memory cell precursors in contrast to CTLs within the clonal burst.

Materials and Methods

Mice

C57BL/6 Spi6 KO and C57BL/6 Spi6 KO P14 TCR 

(Spi6 KO P14) mice have been previously described (25). C57BL/6 CD45.1 congenic mice were purchased from Taconic Farms. All mice were maintained and bred under standard specific pathogen-free conditions. All experiments with mice were performed in compliance with the University of Chicago Institutional Animal Care and Use Committee regulations.

Infections

For primary immune responses, mice were infected by either i.p. injection (2 x 10^3 PFU) of LCMV Armstrong (27) or i.v. injection of L. monocytogenes strain DPL-1942 (10^5 CFU), which has been engineered to express anti-CD8 and the DNA dye YOPRO-1. Tetramer-positive CD8^+ cells were gated and the percentage shown in each plot (upper left corner) and YORPO-1 staining displayed on histograms as the percentage of YOPRO-1^high (upper right corner). B. The mean percentage ± SEM of gp33^+ CD8^+ cells (n = 5 mice) was measured in PBLs, and the mean percentage ± SEM of OVA^+ CD8^+ cells (n = 5 mice) was measured on day 7 (F) or day 15 (G) after infection. B6 or Spi6 KO mice were infected with LCMV Armstrong or L. monocytogenes DPL-1942 OVA (10^6 CFU/mouse i.v.). E. The mean percentage ± SEM of OVA^+ CD8^+ cells (n = 5 mice) was measured in the serum.

Anti-Spi6 antiserum

Recombinant Spi6 was generated in the pGEX-3X expression system in Escherichia coli as a fusion protein with GST and purified to homogeneity (>90% pure), using standard procedures recommended by the manufacturer (Amersham Biosciences). Rabbits were primed with 0.35 mg of recombinant GST-Spi6 in CFA then boosted twice with 0.15 mg then finally with 0.35 mg in IFA.

Flow cytometry

The following mAbs were used: anti-CD8a (allophycocyanin-labeled), anti-CD45.2 FITC or R-PE, anti-CD44 PE, anti-IFN-γ PE (rat IgG1) and rat IgG1-PE isotype control, anti-IL-7R biotin (clone B12-1) then streptavidin-Cy5 (all from BD Pharmingen), and anti-human GrB (clone GB12) then anti-mouse IgG allophycocyanin (both from Caltag Laboratories). H-2D^b tetramers with gp33 (KAVYNFATM) (24, 27) or H-2K^b tetramers with OVA were labeled with streptavidin-PE (Beckman Coulter) (24, 25, 27). PBLs and splenocytes were prepared and stained with tetramers and mAbs as before (24, 27). T cells were enriched by magnetic sorting with anti-thy1.2 beads before purification by FACS (MoFlo; DakoCytomation). Naive cells (CD44^lowCD8^+) were FACS-purified from the spleens of uninfected C57BL/6 mice by staining with anti-CD45.2 FITC or R-PE, anti-CD44 PE, anti-IFN-γ PE, anti-IL-7R biotin (clone B12-1), then streptavidin-Cy5 (both from BD Pharmingen), and anti-human GrB (clone GB12) then anti-mouse IgG allophycocyanin (both from Caltag Laboratories). H-2D^b tetramers with gp33 (KAVYNFATM) (24, 27) or H-2K^b tetramers with OVA were labeled with streptavidin-PE (Beckman Coulter) (24, 25, 27). PBLs and splenocytes were prepared and stained with tetramers and mAbs as before (24, 27). T cells were enriched by magnetic sorting with anti-thy1.2 beads before purification by FACS (MoFlo; DakoCytomation). Naive cells (CD44^lowCD8^+) were FACS-purified from the spleens of uninfected C57BL/6 mice by staining with anti-CD8a allophycocyanin and anti-CD44 PE Abs. Apoptosis of live cells (propidium iodide-negative) was measured with YOPRO-1 dye (green fluorescence; Molecular Probes), as before (30).

Intracellular staining (ICS) for Spi6 was then detected with anti-rabbit IgG-allophycocyanin (Jackson Immunoresearch Laboratories). To detect functional memory cells, splenocytes (5 x 10^6/ml) in 0.2 ml were incubated with either gp33 or OVA peptides (each at 10^{-7} M) for 5 h in the presence of Golgi-block and IFN-γ detected by ICS according to manufacturer’s instructions (BD Pharmingen).

IFN-γ ELISA

Serum (1 ml) was recovered by cardiac puncture and the level of IFN-γ determined in dilutions using a sandwich ELISA according to the manufacturer’s instructions (BD Pharmingen).

Real-time PCR

We generated cDNA (27) and performed real-time PCR (27) using primers and probes used are specific for Spi6 (serpin b9, GenBank accession number U96700) (26) GrB (accession number M12302) (5), and cyclophilin A (31) (forward 5’-CCA TCA AAC CAT TCC TTC TGT AGC-3’. reverse

FIGURE 1. Altered survival of CD8 T cells in Spi6 KO mice. A, Flow cytometric analysis of B6 or Spi6 KO mice on day 8 and day 14 after infection with either LCMV Armstrong (2 x 10^3 PFU/mouse i.p.) or L. monocytogenes DPL-1942-ova (10^5 CFU/mouse i.v.). PBLs were stained with either gp33/H-2D^b or OVA/H-2K^b tetramer with anti-CD8 and the DNA dye YOPRO-1. Tetramer-positive CD8^+ cells were gated and the percentage shown in each plot (upper left corner) and YORPO-1 staining displayed on histograms as the percentage of YOPRO-1^high (upper right corner). B, The mean percentage ± SEM of gp33^+ CD8^+ cells (n = 5 mice) was measured in PBLs, and the mean percentage ± SEM of gp33^+ CD8^+ cells (n = 5 mice) was measured on day 14 after infection. B6 or Spi6 KO mice were infected with LCMV Armstrong or L. monocytogenes DPL-1942 OVA (10^6 CFU/mouse i.v.). C, The mean percentage ± SEM of gp33^+ CD8^+ cells (n = 5 mice) was measured on day 8 or (D) day 14 after infection. B6 or Spi6 KO mice were infected with LCMV Armstrong or L. monocytogenes DPL-1942 OVA (10^6 CFU/mouse i.v.). E, The mean percentage ± SEM of OVA^+ CD8^+ cells (n = 5 mice) was measured in the serum.
5'-AGC AGA GAT TAC AGG ACA TTG CG-3', probe 5'-CAG GAG AGC GTC CCT ACC CCA TCT G-3'. Data were calculated as the ratio of candidate RNA expression per amount of cyclophilin A.

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.

Statistics
The significant difference was measured using a two-tailed Student’s t test.

Results
Spi6 determines the size of the clonal burst
Several studies have supported a linear differentiation model of memory T cell development in which memory T cells are derived

FIGURE 2. The effect of Spi6 on CD8 T cells is cell autonomous. 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, then the mean number of donor LCMV-specific cells (CD45.2+ gp33+ CD8+) (A) and the mean percentage ± SEM of YOPRO-1+ donor cells determined in splenocytes (n = 5 mice) (B). C, The mean concentration ± SEM of IFN-γ (n = 5 mice) measured in the serum of recipient mice after adoptive transfer and LCMV infection.

FIGURE 3. Spi6 and memory CD8 T cells. A, Flow cytometric analysis of spleen cells stimulated ex vivo with peptide Ag 140 days after infection of B6 or Spi6 KO mice with LCMV (i) or 365 days after infection with L. monocytogenes DPL-1942-OVA (ii). Donor CD45.2+ P14 CD8 T cells (10^4) from B6 or Spi6 KO mice were adoptively transferred to B6 CD45.1 recipients, infected with LCMV, then spleen cells stimulated after 150 days. The percentage of IFN-γ+ CD8+ cells is shown (upper left corner) for groups i and ii, and for group iii the percentage of IFN-γ+ CD8+ cells of the CD45.2+ population after gating is shown (upper left corner). In recall experiments, LCMV-infected mice (i and iii) were reinfected on day 320 with the clone 13 variant of LCMV Armstrong (10^6 PFU/mouse i.v.) and L. monocytogenes-infected mice (ii) were reinfected on day 400 with DPL-1942 OVA (10^6 CFU/mouse i.v). After 5 days, the percentage of recall IFN-γ+ CD8+ cells were determined as described. Histograms showing the mean number ± SEM (N = 5 mice) of IFN-γ+ CD8+ memory cells specific for LCMV (B) or L. monocytogenes (C) are represented. D, Histograms showing the mean ± SEM (n = 5 mice) number of CD45.2+ IFN-γ+ CD8+ cells derived from donor P14 CD8 T cells from B6 or Spi6 KO mice are shown. Histograms showing the mean number ± SEM (n = 5 mice) of IFN-γ+ CD8+ recall effectors specific for LCMV (E), L. monocytogenes (F), or LCMV after adoptive transfer of P14 CD8 T cells (G). For LCMV recall responses, the reinfecion of recipients harboring P14 CD8 T cells was the same as intact mice in A.
from effectors (8, 9, 16, 32). Given the role played by Spi6 in protecting CTLs from self-inflicted damage (25), we examined the role of Spi6 in memory CD8 T cell development. We determined the effect of Spi6 on the maximal level of CD8 T cells (clonal burst size) by infecting Spi6 KO and wild-type B6 mice with LCMV Armstrong (27) or the attenuated DPL-1942 strain of L. monocytogenes or L. monocytogenes-specific CTLs in the spleen of Spi6 KO mice, which in turn could be corrected by GrB deficiency (25). We conclude that our present study confirms that Spi6 determines clonal burst size by protecting CTLs from GrB-mediated apoptosis.

Absence of a contraction phase for Spi6 KO CD8 T cells

After the massive increase in the number of effectors that occurs during the expansion phase, an equally dramatic decrease in the numbers of Ag-specific CD8 T cells occurs in the contraction phase (1). Eight days after the infection of B6 mice we observed contraction in the number of LCMV-specific CD8 T cells and a stable level of memory CD8 T cells after 140 days (Fig. 1B). The severity of the contraction phase was measured by comparing the level of LCMV-specific CD8 T cells on day 8 (8 ± 0.6%, n = 5) by the level after 140 days (1.2 ± 0.6%, n = 5). Thus, similar to several previous studies in B6 mice (11, 14, 24, 25), the level of residual tetramer-positive T cell population in the memory phase was 15% of the clonal burst size after LCMV infection (Fig. 1B) and 10% after L. monocytogenes infection (Fig. 1E).

In Spi6 KO mice, we observed a dramatic difference in the severity of the contraction phase. In contrast to B6 mice, there was a low or negligible loss of CD8 T cells in the contraction phase after infection with either LCMV (Fig. 1B) or L. monocytogenes (Fig. 1E). The absence of cell loss in the contraction phase meant that there was no significant difference (p = 0.1) in the level of either LCMV-specific (Fig. 1B) or L. monocytogenes-specific (Fig. 1E) CD8 T cells in the memory phase compared with the maximal clonal burst size level. This stability in the number of Spi6 KO CD8 T cells after the clonal burst correlated with a reduced proportion undergoing PCD, as evidenced by YOPRO-1 staining (Fig. 1A) on day 14 after LCMV (Fig. 1D) and L. monocytogenes (Fig. 1G) infection.

The inflammatory mediator IFN-γ is required for the induction of PCD of CD8 T cells in the contraction phase (20, 21). The diminished induction of PCD of LCMV-specific CD8 T cells during the contraction phase correlated with a reduction of IFN-γ in the serum of Spi6 KO mice during the time of the clonal burst (day 4, Spi6 KO three times lower than B6, p = 2 × 10⁻⁵) and into the contraction phase (day 10, Spi6 KO three times lower than B6, p = 2 × 10⁻⁵) (Fig. 1H). Because CD8 T cells are an important source of IFN-γ after infection (34), we conclude that the reduction in serum IFN-γ and PCD in the contraction phase is likely a result of the diminished clonal burst size in Spi6 KO mice.

Cell autonomous effect of Spi6 on CTL survival

Spi6 can protect DCs from granule-mediated killing by CTLs (35) and so defective priming may have caused the diminished clonal burst size in Spi6 KO mice. Therefore we determined whether the effect of Spi6 on CTL survival was specific to CD8 T cells. Spi6 KO mice were crossed with C57BL/6 P14 transgenic mice, which express a TCR specific for the gp33 peptide Ag of LCMV in the context of H-2Db (36). Naive P14 DC8 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. Analysis of splenocytes revealed that the number of donor LCMV-specific CTLs (CD45.2⁺) from Spi6 KO mice was 7-fold lower (p = 3 × 10⁻⁸) on day 5 by 3-fold (p = 0.01) than B6 P14 CD8 donor CTLs on day 8 (Fig. 2A). As we observed in the whole animal experiments (Fig. 1B), we observed a corresponding 2-fold increase (p = 0.04) in the proportion of donor Spi6 KO CTLs shown that the lower clonal burst size of CD8 T cells in Spi6 KO mice correlated with increased onset of PCD after LCMV (Fig. 1C) or L. monocytogenes (Fig. 1F) infection. These findings are consistent with the decrease in the clonal burst size of LCMV and L. monocytogenes-specific CTLs in the spleen of Spi6 KO mice, which in turn could be corrected by GrB deficiency (25). We conclude that our present study confirms that Spi6 determines clonal burst size by protecting CTLs from GrB-mediated apoptosis.

Figure 4. Spi6 ensures the survival of cytotoxic effectors but not memory cell precursors. Mice were infected with LCMV as in Fig. 1, A. Histograms from flow cytometric analysis of IL-7R expression on gated gp33⁺CD8⁺ cells either on day 8 (effectors) or day 140 (memory) after infection. The vertical broken line indicates the median level of IL-7R expression on naive CD44lowCD8⁺ cells. The horizontal line indicates the population of cells expressing IL-7R above (hi) and below (lo) the mean naive level. The percentage of IL-7Rhi cells is indicated. On day 8 and day 14 after infection, the mean percentage ± SEM (n = 5 mice) of the IL-7Rhi for gp33⁺CD8⁺ cells (B) and the IL-7Rlo of gp33⁺CD8⁺ cells (D), or the mean absolute number ± SEM (n = 5 mice) of the IL-7Rhi for gp33⁺CD8⁺ cells (C) and the IL-7Rlo of gp33⁺CD8⁺ cells (E) was determined.
undergoing apoptosis prior during the clonal burst phase (Fig. 2B). During the contraction phase, the number of donor Spi6 KO P14 CD8 T cells was no different (p = 0.2) than the number of B6 P14 CD8 T cells (Fig. 2A). The restoration of the level of Spi6 KO P14 CD8 T cells to wild-type levels on day 25 of the contraction phase was likely due to the decrease (p = 0.02) in proportion of cells undergoing apoptosis (Fig. 2B). Therefore both the increase in apoptosis during the clonal burst and the decrease after the contraction phase is due to a cell autonomous effect of Spi6 deficiency on CD8 T cells. The expansion and contraction of Spi6 KO P14 CD8 T cells was exaggerated (Fig. 2, A and B) compared with that of endogenous Spi6 KO CD8 T cells (Fig. 1B). This effect is most likely due to an nonphysiologically high precursor frequency of TCR-transgenic CD8 T cells after adoptive transfer, which other studies have shown gives rise to exaggerated and altered kinetics of T cell expansion and contraction (37, 38). However although the adoptive transfer experiments are less physiologically relevant than those with endogenous T cells, they do point to a cell autonomous affect of Spi6 on CD8 T cell survival.

As with the whole animal experiments (Fig. 1G), we observed lower levels of serum IFN-γ in recipients harboring LCMV-specific CD8 T cells from Spi6 KO mice (Fig. 2C). Therefore the reduction in serum IFN-γ as a cell intrinsic defect of Spi6 KO CD8 T cells. We conclude that the absence of cell loss in the contraction phase after infection of Spi6 KO mice is likely due to a reduction in CTL-derived, IFN-γ-driven apoptosis. 

Spi6 and memory CD8 T cells

To examine the function of Spi6 in the development of long-term memory cells, we determined the number of memory CD8 T cells in Spi6 KO mice. We quantified LCMV and memory CD8 T cells, we determined the number of memory CD8 T cells in Spi6 KO P14 CTL. Therefore, the anti-Spi6 antiserum was highly specific for endogenous Spi6.

FIGURE 6. Spi6 is down-regulated in CD8 memory T cell precursors. A, B6 mice were infected with LCMV then spleen cells stained and Spi6 and Grb10 ICS median level of staining (vertical broken line) on gp33– CD8 T-gated cells on day 8 (effectors) or day 756 (memory) after LCMV infection or in naive CD4+CD8+CD8 T cells in uninfected mice is in each panel. B, Median fluorescence from ICS for Spi6 on naive, effector, and memory CD8 T cells. Each diamond is an individual mouse. Horizontal bar is the mean fluorescence from ICS for IL-7Rhigh (n = 5 mice, 609 ± 198) and IL-7Rlow (n = 5 mice, 679 ± 695) day 8 cells. C, Median fluorescence from ICS for Grb10 on naive, CTLs, and memory CD8 T cells. Each diamond is an individual mouse. Horizontal bar is the mean fluorescence from ICS for IL-7Rhigh (Experiment 1, 539 ± 76) and IL-7Rlow (Experiment 2, 301 ± 134) day 8 cells. Expression of Spi6 mRNA (D) and Grb10 mRNA (E) in FACS purified cells measured by real-time PCR. For day 8 cells, IL-7Rlow (lo) or IL-7Rhigh (hi) cells were analyzed. The data are from two independent experiments with n = 5 (■) and n = 10 (□) mice each.
the generation of secondary effectors was autonomous to Spi6 KO CD8 T cells because we observed a 6-fold decrease (p = 2 × 10^-5) in the number donor secondary LCMV-specific CD8 T cells after adoptive transfer of Spi6 KO P14 CD8 T cells (Fig. 3G). We also observed a similar defect in the CD8 recall response in Spi6 KO mice as measured by tetramer staining (data not shown). Because the number of Ag-responsive, primary Spi6 KO memory CD8 T cells was unaffected (Fig. 3, A–D), it would seem very likely that the impaired recall responses are due to a defect in the secondary clonal burst. This in turn is consistent with the requirement for Spi6 to protect CTLs from self-inflicted damage (25).

Survival of memory cell precursors is independent of Spi6
The development of Spi6 KO memory CD8 T cells follows a pathway in which the size of the primary memory pool is not proportional to the size of clonal burst (Figs. 1 and 3). These findings suggest that although Spi6 protect CTLs from PCD, it does not play a role in memory cell development. To test this role we determined whether Spi6 was required for the survival of memory CD8 T cell precursors. We used the expression of IL-7R to identify the small population of CTLs (IL-7R^high) that contain the precursors of memory CD8 T cells (22–24). On d8 after LCMV infection, the percentage IL-7R^high CTLs among gp33^+ tetramer-specific cells was higher in Spi6 KO mice (14%) than B6 controls (6%) (Fig. 4A). Analysis of Spi6 KO mice (n = 5) revealed a significant increase in percentage of IL-7R^high cells at day 8 (p = 2 × 10^-4) and day 14 (p = 0.02) in comparison to B6 mice (n = 5) after infection (Fig. 4B). However, taking into account the smaller clonal burst, the absolute number of IL-7R^high gp33^+ CD8^+ cells in Spi6 KO mice was no different (p = 0.3) from that of wild-type B6 mice (Fig. 4C). Although Spi6 was not required for the survival of memory cell precursors, it was required for the survival of the majority IL-7R^low population, which was reduced by 3-fold on day 8 (p = 4 × 10^-4) and day 14 (p = 0.003) (Fig. 4E). We conclude that Spi6 is required for the survival of the majority of CTLs but not the minority putative memory cell precursor population. Therefore the development of memory CD8 T cells is unaffected in Spi6 KO mice because Spi6 is not required for the survival of memory precursors.

Memory cell precursors express lower levels of Spi6 and GrB
To understand why the survival of memory cell precursors is independent of Spi6 we examined the expression of Spi6 in memory precursor CD8 T cells. ICS with Spi6-specific antiserum (Fig. 5) revealed that Spi6 was up-regulated by 7-fold in day-8 gp33^+ CD8^+ CTLs from wild-type B6 mice compared with naive CD44^low CD8^+ cells from uninfected B6 mice (Fig. 6A). Although the level of Spi6 decreased upon differentiation into memory cells,
it was retained at a level of ~2-fold higher than naive. The physiological target for the suppression of PCD by Spi6 in CTLs is GrB (25, 27) and so we measured expression by ICS. As expected (9), GrB was 266-fold up-regulated in day-8 CTLs compared with naive cells and retained at a low level in memory CD8 T cells (3-fold higher than naive) (Fig. 6A). Therefore, as we have shown for mRNA levels (27), Spi6 is coordinately up-regulated in CTLs along with GrB, thereby affording protection from self-inflicted damage.

Analysis of IL-7R expression revealed that the memory precursor population of gp33^+CD8^+ cells on day 8 after infection expressed lower levels of Spi6. We observed a significant 2-fold decrease in Spi6 median expression (p = 0.009) compared with IL-7R_{low} cells (Fig. 6B). Furthermore, among IL-7R_{high} cells, there was a 2-fold decrease (p = 0.01) in comparison to IL-7R_{low} cells in expression of GrB, the physiological target of Spi6 in CTLs. The decreased expression of Spi6 and GrB in IL-7R_{high} cells in comparison to IL-7R_{low} cells was also observed at the level of mRNA level. Real-time PCR analysis of FACs-purified CD8 T cells revealed that Spi6 mRNA was approximately two times lower (p = 0.001) (Fig. 6C) and GrB mRNA approximately nine times lower (p = 4 \times 10^{-9}) (Fig. 6D) in IL-7R_{high} compared with IL-7R_{low} day-8 CTLs. The down-regulation of Spi6 and GrB in IL-7R_{high} cells would account for the survival of memory cell precursors being unaffected in Spi6 KO mice.

A Spi6_{low}GrB_{low} lineage of memory cell precursors

To determine the cellular basis for the decrease in Spi6 and GrB expression in IL-7R_{high} memory precursors, we examined LCMV-specific CD8^+ cells over time. ICS revealed the presence of a Spi6_{low}GrB_{low} population as early as day 6 after infection of wild-type B6 mice, which became progressively more enriched in the IL-7R_{high} pool over time (Fig. 7A). Analysis of wild-type B6 mice (n = 3) showed a consistently lower proportion of Spi6_{high}GrB_{low} cells in the IL-7R_{high} memory precursor population compared with the IL-7R_{low} population throughout the clonal burst on day 8 (p = 0.001) and the contraction phase on day 16 (p = 0.002) (Fig. 7B). The corresponding analysis of GrB expression also revealed a GrB_{low} population enriched in the IL-7R_{high} pool (Fig. 7A), as evidenced by a significantly (p = 0.003) lower GrB_{high} to GrB_{low} ratio on day 8 (Fig. 7B). By day 12, GrB_{high} cells were rare in both the IL-7R_{high} and IL-7R_{low} populations (Fig. 7). The level of Spi6 and GrB expression that marked the Spi6_{low}GrB_{low} (two times higher than naive level) and GrB_{low} (three times higher than naive level) populations was the same as that in the stable memory pool. We conclude that the reduction in Spi6 and GrB expression in memory cell precursors in the clonal burst is due to the presence of an enriched Spi6_{low}GrB_{low} population. During the contraction phase IL-7R_{high}Spi6_{low}GrB_{low} memory precursors increase in proportion presumably because they are refractory to the self-inflicted death that results in the removal of the majority IL-7R_{low}Spi6_{high}GrB_{high} population.

Discussion

It is widely accepted that after infection the size of the clonal burst determines the size of the population of memory CD8 T cells (10–15). As we have reported previously (25) and confirmed in the present study, infection of Spi6 KO mice with LCMV or L. monocytogenes results in a drastically diminished clonal burst because Spi6 deficiency renders the majority GrB_{high} population of CTLs susceptible to PCD (Fig. 8). The degranulation of CTLs prevents the development of memory CD8 T cells (30) and so memory precursors are not intrinsically resistant to GrB. Therefore, it is likely that memory cell precursors survive in Spi6 KO mice because they express low levels of GrB. In contrast, the majority nonmemory population of CTLs, which expresses high levels of GrB and Spi6, fail to survive in Spi6 KO mice (Fig. 8). The development of wild-type Spi6 memory cells is accompanied IFN-γ-driven PCD of clonal burst CTLs, which leads to the characteristic loss of cells during the contraction phase (20) (Fig. 8). For Spi6 KO CD8 T cells, the development of memory cells occurs when the diminished clonal burst of CTLs results in less IFN-γ-driving PCD and the absence of cell loss in the contraction phase. Spi6 protects the majority wild-type IL-7R_{high}GrB_{high} population of CTLs from PCD but is not required to protect the IL-7R_{low}Spi6_{low}GrB_{low} population of memory precursors from PCD. Therefore, even when the clonal burst size is severely diminished...
in Spi6 KO mice the size of the memory precursor pool is affected and so memory cell development is unimpacted (Fig 8).

The pathway of primary memory CD8 T cells development we describe in Spi6 KO mice is also seen in other instances when IFN-γ levels are reduced either in IFN-γ KO mice (20) or when inflammation is curtailed by antibiotics in L. monocytogenes-infected, wild-type mice (21). Reduced IFN-γ causes a decrease in the clonal burst and the development of primary memory cells without a contraction phase (20). However, it is unclear why reduced IFN-γ levels alone should diminish clonal burst size. In our study, it is clear that Grb-mediated PCD decreases the size of the clonal burst in Spi6 KO mice (25). Although reduced levels of IFN-γ has been shown to increase the proportion of IL-7R^high memory precursors (21), the effect on the absolute number was not tested in previous studies so they did not directly address the question of whether memory cell precursors are susceptible to IFN-γ-induced PCD. In Spi6 KO mice, despite reduced levels of IFN-γ there is no difference in the absolute number of memory cell precursors (Fig 4) or primary memory cells (Fig 3). So we can conclude from our present study that memory cell precursors are refractory to PCD associated with IFN-γ. Thus, neither the Grb nor IFN-γ effector molecules play a role in selecting memory cell precursors, in agreement with earlier findings of wild-type levels of memory CD8 T cells after infection of perforin KO (20), IFN-γ KO (20), or Grb KO mice (39). Rather we conclude that effector molecules are important in regulating clonal burst size by inducing the PCD of CTLs. It is likely that the residual expression of Spi6 in long-term memory CD8 T cells, which retain Grb, is required for the generation of recall CTLs rather than for maintaining primary memory cells (Fig 3). The specific requirement for Spi6 in determining the clonal burst of CTLs but not the development of memory CD8 T cells is consistent with recent studies with mice deficient in the Id2 transcriptional regulator, which is required for Spi6 up-regulation (40).

The linear differentiation model of memory T cell development predicts that memory T cells at some point are derived from CTLs (8, 9, 16, 32). The enrichment of the IL-7R^high population with CD8 T cells with reduced Grb expression seems to suggest that memory precursors are not fully differentiated effectors. However, the expression level of Grb and Spi6 in memory precursors is still twice that of naive CD8 T cells and equivalent to memory CD8 T cells (Figs 6 and 7). In addition, memory CD8 T cells express the gene encoding the Fas ligand cytotoxic molecule at a higher level twice that of naive CD8 T cells and equivalent to memory CD8 T cells (Fig. 7). Thus, neither the Grb nor IFN-γ effector molecules play a role in selecting memory cell precursors and Grb^KO Spi6^KO CTLs come from the analysis of Grb expression throughout CD8 T cells expansion and contraction after LCMV infection. Although there is a discrete population of Grb^low cells as early as day 6 after LCMV infection, the proportion of these cells decreases on day 8, then increases sharply during the contraction phase on day 12 and day 16 (Fig. 7A). Therefore, we do not think that the Grb^low population we propose as memory cell precursors represents a lineage of non-effectors that differentiate into memory cell in parallel line-to-effectors because if this were so we would expect the proportion to increase from day 6 to day 8. Rather, it would seem likely that the development of the Spi6^lowGrb^low memory precursor population is due to the down-regulation of Grb after day 8 preferentially in IL-7R^high cells and the possible enrichment for Grb by escape from PCD. Because lowered Spi6 expression would serve no conceivable advantage for CTLs avoiding PCD, we propose that it is actively down-regulated in IL-7R^highGrb^low cells. Examination of the expression of the perforin effector molecule in TCR transgenic CTLs in vitro did not reveal an equivalent perforin^low population (8). This may be because perforin^low memory cell precursors may only develop under the same conditions that produced Grb^KO cells, that is to say in vivo activation by infection from a physiological level of naive precursors that express endogenous TCRs. Our findings with CD8 T cells are consistent with those with Th1 CD4 T cells, which indicate that memory cells are derived from effectors that express low levels of IFN-γ (41).

In summary, we provide evidence that the development of memory CD8 T cells requires the suppression of biochemical pathways distinct from those that control the size of the clonal burst of CTLs. An attractive strategy to provide immunity to some viruses is to enhance CTL function by protection from PCD. Our findings give a molecular explanation for the independence of memory cell development from survival factors that govern clonal burst size and in turn predict that successful vaccines that give rise to long-term memory will do more that just protect CTLs from death.

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Disclosures

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