

Novel vaccine strategies to protect against intracellular pathogens, particularly those for which antibodies alone are ineffective, focus on inducing memory CD8 T cells—the long-lived counterparts of cytolytic effector T cells. Upon acute infection of host tissue by an intracellular pathogen, naïve CD8 T cells (T_N) proliferate and differentiate into effector T cells (T_E), that then contract by apoptosis leaving behind a small surviving population that progressively differentiates into memory T cells (T_M). The long-term maintenance of the memory population occurs through balanced cell division and cell death, a process termed homeostatic proliferation.

The extrinsic and intrinsic factors that determine the selection and maintenance of CD8 T_M are poorly understood. Recent studies of CD8 T cell differentiation have identified transcripts that are differentially expressed in CD8 T_M . One candidate gene identified by these studies is Bmi-1, a transcription factor which has been termed the “master regulator” of hematopoietic stem cell (HSC) maintenance. Murine *bmi1*^{-/-} adult HSCs exhibit both survival and proliferation defects associated with dysregulated expression of multiple genes involved in apoptosis and cell cycle control, leading to a progressive loss of all hematopoietic lineages and death of *bmi1*^{-/-} mice by two months of age. To better understand the cell intrinsic mechanisms that select CD8 T_M during the contraction phase and govern their long-term maintenance, we propose analyzing the function of Bmi-1 in both differentiating and established CD8 T_M populations.

Specific Aim 1: Determine the role of Bmi-1 in the death of activated CD8 T cells. Since Bmi-1 regulates the expression of anti-apoptotic genes, and its expression is increased in differentiating CD8 T_M , we hypothesize that Bmi-1 is involved in protecting a fraction of the Ag-specific CD8 T_E population from apoptosis during the contraction phase. This hypothesis will be tested using a retroviral transduction system to ectopically express or knockdown Bmi-1 in activated CD8 T cells. The kinetics of expansion and contraction will be analyzed in Bmi-1-manipulated CD8 T cells activated both *in vitro* and *in vivo*. Additionally, the survival of activated, Bmi-1-manipulated CD8 T cells will be analyzed under specific apoptosis-inducing conditions, using flow cytometric detection of apoptotic markers.

Specific Aim 2: Determine the role of Bmi-1 in the homeostatic proliferation of Ag-specific CD8 T_M . Because Bmi-1 controls the self-renewal of HSCs, and increased *bmi-1* mRNA levels are found in CD8 T_M , we hypothesize that it is similarly involved in the self-renewal of CD8 T_M . To analyze the long-term maintenance of an Ag-specific CD8 T_M population generated *in vivo*, fetal liver chimeras will be made by retroviral transduction of *bmi-1* cDNA into HSCs, and adoptive transfer of positively-transduced cells to congenic, lethally-irradiated hosts. After re-constitution, Bmi-1 chimeras will be infected with LCMV Armstrong, and rested to generate LCMV-immune mice. Quantitation of Ag-specific CD8 T_M will be combined with BrdU labeling to analyze the effects of

Bmi-1 over-expression on CD8 T_M survival and proliferation. In addition, endogenous Bmi-1 expression will be analyzed in cycling and non-cycling Ag-specific CD8 T_M populations from B6-P14 LCMV-immune mice using quantitative RT-PCR.

I. Significance

Memory CD8 T cells are more effective at eliminating infection with intracellular pathogens than their naïve counterparts (1). Although CD8 T_M can be rapidly identified, purified, and studied using current technologies, little is known about the cell-intrinsic factors which guide their differentiation and maintenance. Besides contributing to the general understanding about cell fate choices in dynamic lymphocyte populations, a complete understanding of these processes at the molecular level will hopefully provide targets for manipulation. Specifically, therapies for cancer and HIV involve the *ex vivo* activation of CD8 T cells, and subsequent manipulation with recombinant viral vectors (2-4). Such techniques could potentially benefit from the inclusion of genes in these expression vectors which increase the survival of CD8 T cells both *in vitro* and *in vivo*, or which promote the differentiation of functional, long-lived CD8 T_M. Additionally, manipulation of factors which promote CD8 T_M differentiation and maintenance have implications for vaccine design, particularly current approaches aimed at generating protective, Ag-specific CD8 T cell populations (5, 6).

Furthermore, the observation that CD8 T cells selected into the memory lineage re-gain the property of self-renewal characteristic of their HSC precursors, suggests that the specific factors which confer this property may overlap. An unbiased analysis of gene expression patterns in these two cell types is consistent with this hypothesis, and further characterization of the key regulators involved should yield a better understanding of how lymphocyte life-spans and population dynamics are controlled (7).

II. Background

Recent data suggests that the generation of CD8 T_M after acute viral infection is a gradual process (8, 9). The end result is a population of CD8 T_M that upon secondary infection are more effective at eliminating the pathogen than naïve CD8 T cells of the same specificity (10). This enhanced protection is due in part to the fact that following primary infection, antigen (Ag)-specific CD8 T_M are stably maintained at higher frequencies than their naïve counterparts (11,12). The process of differentiation from T_N cell to T_M cell is an area of active investigation, and a number of different models for this process have been proposed (6). Several studies from acute viral infections of mice are consistent with the idea that a fraction of the Ag-specific CD8 T_E population selectively survives the contraction phase and establishes the CD8 T_M population (13-15). For example,

following adoptive transfer of *in vitro*-activated TCR Tg CD8 T cells with potent CTL activity to naïve mice, a long-lived CD8 T_M population develops that responds robustly to antigenic challenge (16).

The dramatic purging of the activated T cell repertoire that occurs following pathogen clearance implies that a balance exists between the actions of pro-survival and death-inducing molecules in differentiating CD8 T_M, likely in response to both environmental cues and cell-intrinsic transcriptional programs. Specifically, it appears that activated T cells can receive at least two different types of apoptosis-inducing signals (17-19). One is extrinsically-mediated and is characterized by signaling through receptors of the tumor necrosis factor (TNF) superfamily such as Fas and TNFRI on the surface of activated T cells (20-22). This results in an intracellular signaling cascade within the T cell that culminates in caspase-dependent apoptosis. Importantly, this pathway is dependent on repeated stimulation of activated T cells through the TCR, and is independent of both protein synthesis and inhibition by anti-apoptotic members of the B-cell lymphoma (Bcl) family (17, 23, 24). Many early studies identified a critical role for Fas and TNFRI in activated T cell death *in vitro* (20, 22, 23, 25). However, mice with loss-of-function mutations in Fas and TNFRI exhibit no net increase in CD8 T cells following acute viral infection, therefore the exclusive roles of these death receptors in CD8 T cell contraction is questionable (26-29).

Alternatively, activated T cells that lack TNF family member receptors are susceptible to intrinsic apoptosis triggered by stresses such as growth factor deprivation (17). In this case, cell death is dependent on new protein synthesis, independent of repeated TCR stimulation, and results from a shift in the balance between pro and anti-apoptotic members of the Bcl family (30, 31). There is equivocal data which supports a role for specific Bcl family members in activated T cell death following pathogenic infection (32-34). Clarifying the contribution of both extrinsic and intrinsic apoptotic triggers to the death of CD8 T_E, specifically, will aid in understanding the generation of CD8 T_M following acute infection. Along with identifying the molecules that directly mediate the death or survival of activated CD8 T cells, it will be important to study how the expression and function of these mediators are regulated.

Although the details of how CD8 T_M are generated following pathogen clearance are unclear, it is known that contraction results in a stable pool of long-lived CD8 T_M (11). In addition to survival, CD8 T_M maintenance is marked by slow proliferation (35). When Ag-specific CD8 T_M are examined at steady state, the majority of the population is quiescent and non-cycling, as indicated by minimal incorporation of a DNA-labeling dye. Interestingly, a small fraction of the population is in cycle, and this population is enriched in the bone marrow (36). Using microarray chips specific for cell cycle regulatory genes, a recent study found differences in the transcriptional profile between the cycling and non-cycling CD8 T_M populations. Specifically, when the non-cycling cells were

purified, they expressed higher levels of cell cycle inhibitory genes such as p16 and p19, compared to the un-sorted bulk populations (37). Although this finding is not surprising, it suggests that factors which regulate genes such as p16, p19, and cyclins, might also regulate the balance between the two pools of CD8 T_M cells, and thus proliferation itself.

The characterization of the renewal property of CD8 T_M populations combined with the fact that these populations are maintained at constant numbers for the lifetime of an animal, led to the hypothesis that CD8 T_M might be subject to similar extrinsic and intrinsic regulatory factors as their long-lived progenitor, the hematopoietic stem cell (HSC) (6). The observation that both populations share an anatomical niche in bone marrow, suggests that there may be overlapping extrinsic factors which support the survival and renewal of HSCs and CD8 T_M. Furthermore, when the transcriptional profile of these two purified populations was compared, it was found that certain genes which were highly expressed in HSCs were similarly expressed in CD8 T_M. Interestingly, the expression of these genes increased as CD8 T cells differentiated from the naïve to the effector to the memory stage (7). One gene whose expression pattern fits this profile is Bmi-1, a transcriptional regulator of the Polycomb group-- best-characterized for their role in regulation of the Polycomb chromosomes in *Drosophila melanogaster*.

Bmi-1 was originally identified in mammalian studies as an oncogene that can cooperate with *c-myc* in retroviral tagging models of lymphomagenesis (38, 39). However, Bmi-1's cell cycle regulatory properties were not fully appreciated until a *bmi-1*^{-/-} mouse line was generated (40). These mice exhibit severe hematopoietic defects, manifested by the progressive loss of all myeloid and lymphoid lineages in the adult. T and B cells are particularly affected by the lack of Bmi-1, as *bmi-1*^{-/-} mice have four-fold fewer lymphocytes than wild-type. Park and colleagues found that the hypocellularity in the *bmi-1*^{-/-} mice was due to functional defects in the HSC compartment (41). HSCs which lack Bmi-1 exhibit both survival and proliferation defects (41, 42). Although these defects are not yet fully characterized at the molecular level, Bmi-1 expression directly correlates with the expression of anti-apoptotic genes, and inversely correlates with the expression of cell-cycle inhibitory genes in murine bone marrow (41, 43). Specifically, many studies have shown that Bmi-1 negatively regulates the genes p16 and p19, which as mentioned above, are differentially regulated in cycling and non-cycling CD8 T_M (44-48).

Although there are many factors which likely contribute to the regulation of HSCs, Bmi-1 is a key factor required for HSC survival and self-renewal. Now, microarray analyses indicate that Bmi-1 is enriched in long-lived CD8 T_M versus shorter-lived CD8 T_E. Both HSCs and CD8 T_M are found in the bone marrow, and the frequency of cycling CD8 T_M in this population is high. Furthermore, cell cycle inhibitory genes regulated by Bmi-1 are differentially expressed in cycling and quiescent CD8

T_M. Since both anti-apoptotic and cell cycle regulatory genes are linked to Bmi-1, we propose that Bmi-1 has a pro-survival and/or cell-cycle regulatory role in CD8 T_M. To explore this possibility Bmi-1 expression will be manipulated in CD8 T cells and the consequences of this manipulation on (1) the death of activated CD8 T cells and (2) the maintenance of Ag-specific CD8 T_M, will be investigated.

Specific Aim I: Determine the role of Bmi-1 in the death of activated CD8 T cells.

Rationale:

Bmi-1 is necessary for HSC maintenance and its ablation results in the decrease in expression of anti-apoptotic genes in murine bone marrow, and the loss of all hematopoietic lineages. Bmi-1 is also expressed in mature lymphocytes, and its expression increases specifically in CD8 T cells as they differentiate to the memory stage. Therefore, we hypothesize that Bmi-1 has a protective role during the programmed cell death of activated CD8 T cells.

Experimental Design:

Bmi-1 expression will be manipulated using recombinant retroviruses to deliver ectopic *bmi-1* for over-expression, or a *bmi-1* short-hairpin (shRNA) for knock-down, in hematopoietic cells. The murine stem cell virus (MSCV) vector contains transcriptional regulatory elements that have been optimized for use in hematopoietic cells. Integration of the provirus into the host cell genome results in heritable transgene expression that is necessary for stable manipulation of gene expression in our study of rapidly dividing and differentiating lymphocytes (49).

A cDNA which encodes for the full-length Bmi-1 protein, or a *bmi-1* shRNA, have been inserted into separate MSCV vectors. The shRNA sequence is identical to a validated *bmi-1* hairpin that targets the 3'UTR of the *bmi-1* transcript. The MSCV long terminal repeat (LTR) sequences will drive transcription of the *bmi-1* cDNA in the integrated proviral genome, to deliver ectopic Bmi-1 to cells, and this virus is referred to as **Bmi-1(EC)**. For expression of the *bmi-1* shRNA, a human RNA Pol III U6 promoter will drive transcription of the shRNA sequence in the integrated proviral genome and this virus is referred to as **Bmi-1(KD)**. As a control for specific knockdown, a virus expressing a scrambled *bmi-1* shRNA will be made and referred to as **Bmi-1(siSCRAM)**. Immediately downstream of the insert sequence in all constructs is the coding region of the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES) followed by the coding sequence of *thy1.1*, a surface protein used to identify positively transduced cells. This vector design results in collinear transcription of the inserted transgene and *thy1.1* reporter gene from the integrated pro-viral genome, and therefore increases the likelihood that transduced cells which are translating the reporter protein are also translating the inserted transgene. Additionally, a control

virus which expresses only the Thy1.1 reporter will be used in all assays and is referred to as **MiT** (*MSCV-ires-thy1.1*).

A co-transfection system is used for transient production of high-titer retroviruses in HEK 293T cells (50; 293T: ATCC#CRL 11258). Our set of plasmids encode a packaging signal and either *bmi-1-ires-thy1.1*, or *bmi-1shRNA-ires-thy1.1* sequences, flanked by the MSCV LTRs. A separate “helper” plasmid encodes the viral proteins required for packaging and replication in trans (Gag, Pol, and Env) (50) This method reproducibly yields titers of $1-2 \times 10^6$ IU/mL when the viral supernatants are titered on NIH-3T3 murine embryonic fibroblast (MEF) cells (MEFs, ATCC#: CRL-2752). NIH-3T3 MEF cells also constitutively express Bmi-1 and are permissive for infection by the recombinant viruses, thus they will be used to test the efficacy of Bmi-1 manipulation. MEFs transduced with either MiT, Bmi-1(EC) or Bmi-1(KD) will be sorted for Thy1.1 expression on day 3 post-transduction, using the MACS magnetic bead method. Preliminary experiments have shown that 30-70% transduction of MEFs, and >95% purity of sorted Thy1.1⁺ MEFs are achieved using this method (see Fig 1). Quantitative reverse-transcriptase PCR and Western blots on cell lysates from positively transduced cells will be performed to test over-expression and knockdown of Bmi-1 mRNA and protein. Viruses which successfully manipulate Bmi-1 expression in MEFs will also be tested in T cells to ensure similar effects.

Both *in vitro* and *in vivo*, Bmi-1 expression will be manipulated using Bmi-1(EC) and Bmi-1(KD). For the former, splenocytes and lymphocytes will be harvested from C57/BL6 (B6) or P14 TCR Tg mice. The P14 mice express transgenes for the V α 2 and V β 8.1 chains specific for the complex of MHC I-D^b/gp33 peptide from LCMV-- thus the mature lymphocytes are predominantly gp33-specific CD8 T cells (51). The B6 or P14 T cells will be activated *in vitro* by TCR cross-linking with α -CD3 + α -CD28, or by gp33 peptide, respectively. It is necessary that the T cells be cycling for the recombinant virus to integrate into the genome. Positively-transduced cells will be sorted for Thy1.1 expression on day two post-activation, by α -Thy1.1 MACS purification, and then used for the experiments detailed below.

For *in vivo* experiments, mixed fetal liver chimeras will be made by transduction of fetal liver cells from B6 (Ly5.1, Thy1.2) mice with the Bmi-1(EC) virus, using a method adapted from Schmitt et al (52). After four days of *ex vivo* transduction, the fetal liver cells will be sorted by α -Thy1.1 MACS purification. One-million total cells (at a ratio of 60% Thy1.1⁺ cells to 40% non-transduced cells) will be injected into lethally irradiated, adult congenic B6 (Ly5.2) hosts. When chimeric Bmi-1(EC) mice are analyzed at 5-6 weeks post-transfer, 20-30% of peripheral blood CD4 and CD8 T cell populations are positive for the Thy1.1 reporter using this method, and the ratio of CD4 to CD8 T cells in the peripheral blood and spleen are comparable to the MiT control chimeras (see Fig 2).

The following experiments test whether Bmi-1 is involved in protecting activated CD8 T cells from programmed cell death. We expect that if Bmi-1 has a pro-survival function in CD8 T cells, that any changes induced by increasing or decreasing Bmi-1 expression will be manifested as quantitative changes in the number of CD8 T cells during the contraction phase. Therefore, the effects of Bmi-1 manipulation will be analyzed in the context of acute infection with lymphocytic choriomeningitis virus (LCMV- Armstrong strain), as it is one of the best-characterized CD8 T cell responses to a model of murine viral infection.

A. Expansion and contraction of Bmi-1-manipulated CD8 T cells *in vitro*.

To determine if Bmi-1 has an effect on the kinetics of Ag-specific CD8 T cell expansion and contraction, Bmi-1 will be over-expressed and knocked down in P14 T cells, using Bmi-1(EC) or Bmi-1(KD), respectively. P14 T cells transduced with MiT or Bmi-1(siSCRAM) vectors will be the negative controls for non-Bmi-1-mediated effects. P14 splenocytes and lymphocytes transduced with each virus will be cultured separately. After two days, the CD8⁺Thy1.1⁺ cells from each culture will be positively selected using MACS beads, and re-seeded separately. The number of P14 T cells in each culture will be determined on successive days by hemocytometer counting of total cells-- those that both exclude (live) and internalize (dead) the vital dye trypan blue. In addition to comparisons of total cell numbers, differences in proliferation between the cultures will be compared, by labeling of cells directly *ex vivo* using carboxyfluorescein diacetate succinimidyl ester (CFSE), a membrane permeable dye which binds irreversibly to free amines inside the cell. Each time a cell divides its CFSE fluorescence is halved, and thus the number of divisions a cell has undergone can be tracked over time. An aliquot from each culture will be analyzed for CFSE fluorescence at all time points.

Possible results and Interpretations

Our previous work with primary murine CD8 T cells cultured *in vitro* has found that non-transduced CD8 T cells activated using α -CD-3 and α -CD28 mAbs to cross-link the TCR and enhance co-stimulation, respectively, divide and reach a maximum number at day five post-activation. The numbers decline on days 6-8. We expect to observe the same trend for P14 T cells transduced with control MiT and Bmi-1 siSCRAM viruses. Therefore, the numbers of P14 T cells observed in these cultures will be used as a baseline for comparison to Bmi-1-manipulated P14 T cells. To control for possible effects of Bmi-1 manipulation on the expansion of activated CD8 T cells, the numbers of P14 T cells will be examined kinetically to determine the peak of expansion in each culture, and the numbers of P14 T cells on subsequent days will be normalized to these values. In Bmi-1-manipulated P14 T cells, the following results are possible:

(1) *Bmi-1 expression directly correlates with the number of activated P14 T cells observed on days 6-8.* We hypothesize that there will be a greater number of cells in P14 cultures over-expressing Bmi-1 as compared to control P14 cultures on days 6-8. Similarly, we expect that knocking down Bmi-1 expression will result in fewer cells compared to control cultures on days 6-8. These results would suggest that Bmi-1 has a protective role in regulating activated CD8 T cell contraction. This conclusion would be strongly supported if this result is also observed *in vivo* (discussed in Part B).

(2) *Bmi-1 expression inversely correlates with the number of P14 T cells on days 6-8.* Specifically, there could be fewer P14 T cells in cultures over-expressing Bmi-1, and more P14 T cells in cultures where Bmi-1 is knocked down, compared to control cultures on days 6-8. Such results would suggest that Bmi-1 induces the death of activated CD8 T cells. To further test this hypothesis, Bmi-1-manipulated and control P14 T cells would be analyzed at each day post-activation for expression of apoptotic markers. In addition, the expression of known Bmi-1 target genes involved in cell cycle regulation would be analyzed in Bmi-1-manipulated and control cells by quantitative RT-PCR.

(3) *Bmi-1 manipulation in only one direction correlates with quantitative differences in the number of P14 T cells on days 6-8:*

a. Bmi-1(EC) ⇒no difference; Bmi-1(KD) ⇒ decrease in number of P14 T cells on days 6-8.

This result would suggest that Bmi-1 has a pro-survival function in activated CD8 T cells, but that it is not the limiting factor for survival of activated CD8 T cells *in vitro*, and therefore its over-expression alone would not confer enhanced survival. This role of Bmi-1 during P14 T cell contraction would then be tested *in vivo*, either by adoptive transfer of Bmi-1(KD) P14 T cells two days after *in vitro* activation and transduction, or by infection of Bmi-1(KD) chimeras with LCMV (discussed under *Limitations*).

b. Bmi-1(EC) ⇒no difference; Bmi-1(KD) ⇒increase in number of P14 T cells on days 6-8.

This result would suggest that Bmi-1 promotes the death of activated CD8 T cells, but that it is not the limiting factor for this effect *in vitro*, since increasing its expression does not result in more death. This role of Bmi-1 during P14 T cell contraction would then be tested *in vivo*, either by adoptive transfer of Bmi-1(KD) P14 T cells shortly after *in vitro* activation and transduction, or by infection of Bmi-1(KD) chimeras with LCMV (discussed under *Limitations*).

c. Bmi-1(EC) ⇒increase in number of P14 T cells on days 6-8; Bmi-1 KD ⇒no difference.

This result would suggest that Bmi-1 can protect activated CD8 T cells from apoptosis *in vitro*, but that it is redundant, and therefore its function in this context can be performed by other proteins. If similar results are seen *in vivo*, it would indicate that Bmi-1 is not a central regulator of activated CD8 T cell

survival. However, this result could be investigated for possible therapeutic applications, especially those that utilize retroviral therapy for manipulation of human hematopoietic cells *ex vivo*.

d. Bmi-1(EC) ⇒ decrease in number of P14 T cells on days 6-8; Bmi-1 KD ⇒ no difference.

This result would suggest that Bmi-1 can induce the death of activated CD8 T cells, but that it is redundant, and therefore its function in this context can be performed by other proteins, since P14 T cells with Bmi-1 expression knocked down die at the same rate as control P14 T cells. Alternatively, Bmi-1 over-expression could be toxic to activated CD8 T cells. However, based on our preliminary results with Bmi-1(EC) chimeras, Bmi-1 over-expression does not appear to be toxic to naïve or effector CD8 T cells in these mice (discussed under *Limitations*).

(4) *Bmi-1 manipulation affects both the expansion and contraction of activated P14 T cells.*

Although differences in expansion will be standardized to allow for a comparison of contraction between cultures, differences in proliferation observed due to Bmi-1 manipulation would be interesting in light of the cell-cycle regulatory effects of Bmi-1. This could be an area for further study, but is beyond the scope of the experiments discussed in this proposal.

Limitations and Alternative Approaches

Correlation of reporter gene expression with transgene expression. Although the design of the retroviral vector is such that a polycistronic transcript of *bmi-1* cDNA or *bmi-1* shRNA linked to the *thy1.1* reporter gene is predicted, translation of the Bmi-1 mRNA and knockdown efficiency are independent of Thy1.1 translation. Therefore, Thy1.1⁺ cells from each culture would be analyzed for successful over-expression and knock down of Bmi-1 at the beginning and end of the assay, using semi-quantitative (end-point) RT-PCR, or quantitative RT-PCR.

Analysis of CD8 T cells activated in vitro vs. in vivo. It is likely that *in vitro* activation does not faithfully re-capitulate all of the processes that occur during CD8 T cell activation *in vivo*. To explore this possibility, *in vivo* experiments with Bmi-1(EC) chimeras will be done in Part B of this aim.

In addition, it is possible that the death of activated CD8 T cells *in vitro* occurs by different mechanisms than *in vivo*, and therefore no effect of Bmi-1 manipulation will be observed. An alternative is to allow the cells to contract *in vivo*. Specifically, the CD8⁺Thy1.1⁺ cells from Bmi-1-manipulated cultures or control cultures would be positively selected by MACS beads at day two post-activation and transduction. An equal number of positively-sorted cells from each culture would be adoptively transferred into separate groups of congenic, naïve B6 mice. Five to twelve days after transfer (day 8-15 post-transduction and activation) the number of donor P14 T cells in the spleen and lymph nodes would be quantitated. As a control for engraftment of the donor cells, the numbers of donor cells in the spleens and lymph nodes of each group would be analyzed at 24h post-transfer to determine transfer efficiency, and numbers recovered at later time points would be

normalized to these values. The donor P14 T cells would also be labeled with CFSE during the initial transduction and activation step to control for differences in proliferation between Bmi-1-manipulated and control cells after adoptive transfer. Published data suggests that P14 T cells activated *in vitro* with synthetic gp33 peptide will divide significantly and be able to express effector functions following transfer into naïve mice; though it is not clear if P14 T cells activated in this way divide to the same extent and with the same kinetics as P14 T cells activated *in vivo* (1, 16).

B. Expansion and contraction of Bmi-1-manipulated CD8 T cells *in vivo*.

Bmi-1(EC) mixed chimeras will be infected with 2×10^5 pfu of LCMV Armstrong. Then 5, 8, 15, 20, and 30 days post-infection, blood will be collected from the tail veins of infected mice. The frequency of Ag-specific CD8 T cells in the Thy1.1⁺ and Thy1.1⁻ populations will be analyzed by D^b/gp33 MHC I tetramer staining. LCMV-infected MiT mixed chimeras will be used as a negative control for Bmi-1 manipulation, and non-transduced mice will be used as a control for potential retroviral effects on Ag-specific CD8 T cell expansion and contraction.

Possible results and Interpretations

Since Bmi-1 positively regulates the expression of anti-apoptotic genes, we predict that during the contraction phase there will be an increased number of Ag-specific cells in the CD8 Thy1.1⁺ population of Bmi-1(EC) mice compared to MiT and non-transduced controls. Importantly, in the Bmi-1(EC) chimeras, the increased frequency of Ag-specific CD8 T cells should only be in the Thy1.1⁺ and not the Thy1.1⁻ population. While this would not prove that the effect of Bmi-1 was specific only to CD8 T cells, it would suggest that the increased frequency of Ag-specific CD8 T cells was not an indirect effect of ectopic Bmi-1 expression in an accessory cell (such as CD4⁺ T cell or a DC). Increased survival of the Bmi-1(EC) CD8 T cells would suggest that Bmi-1 positively regulates the survival of Ag-specific CD8 T cells during the contraction phase of the LCMV response. Future experiments would focus on identifying the targets of Bmi-1 in activated CD8 T cells. This could be done by performing ChIP analysis on Ag-specific CD8 T cells during and following infection of B6 mice with LCMV, using an α -Bmi-1 mAb. The immunoprecipitated DNA could be digested, and either ligated into sequencing plasmids, or used as a probe for microarray analysis on microchips enriched for murine promoter sequences.

Fewer Ag-specific CD8 T cells in the CD8 Thy1.1⁺ population of Bmi-1(EC) mice during contraction as compared to MiT mice would suggest that Bmi-1 has a death-inducing role. If our other experiments are consistent with this possibility, then we would first verify that apoptosis was occurring by staining cells from Bmi-1(EC) chimeras for apoptotic markers either *ex vivo* or *in situ* at early time points post-infection, and detecting apoptotic CD8 T cells by flow cytometry or TUNEL,

respectively. However, based on what is known about Bmi-1 function, this pro-apoptotic role is likely to be secondary to possible toxic effects of ectopic Bmi-1 expression in CD8 T cells (discussed under *Limitations*).

If no difference between Bmi-1(EC) and control CD8 T cells is observed, it does not disprove our hypothesis, as it is possible that ectopic Bmi-1 is not the limiting factor for CD8 T_E survival *in vivo*. To determine if Bmi-1 has any role in CD8 T_E contraction *in vivo*, we would make fetal liver chimeras using Bmi-1(KD) virus. Due to the severe hematopoietic defects in *bmi-1*^{-/-} mice, it is possible that there will only be transient hematopoiesis in these mice. If the chimeras are made on a TCR Tg background however, there may be enough mature peripheral CD8 T cells to adoptively transfer to a naïve host. The B6-Bmi-1(KD) chimeras would then be infected with LCMV, and the donor CD8 T cells analyzed as described above.

Limitations and Alternate Approaches

The main limitation to the chimera approach is that the strong LTR promoter will likely result in the ectopic expression of Bmi-1 in all hematopoietic cells, which could complicate interpretations of specific effects of Bmi-1 over-expression on CD8 T cells. However, the inclusion of non-transduced fetal liver cells in the mixed chimera partially controls for this, as any apparent effect of ectopic Bmi-1 expression on CD8 T cells that might be caused indirectly by the effects of Bmi-1 on accessory cells, should be manifested in the non-transduced CD8⁺Thy1.1⁻ cells.

The experiments outlined above propose to examine Ag-specific CD8 T cells in the blood longitudinally. For all of the possible outcomes- increased, decrease or no change in the number of Ag-specific CD8 T cells-- it is possible that there was a skewed distribution of Ag-specific CD8 T cells into or out of the blood, due to the existence of heterogeneous populations of CD8 T cells that express different homing molecules following acute viral infection (53). This would be tested by analyzing the frequency of Ag-specific CD8 T cells in both lymphoid and peripheral tissues of infected chimeras and control mice at the indicated time points.

Any quantitative differences between the Ag-specific CD8 T cell populations in Bmi-1(EC) and control chimeras could be due to differences in proliferation, survival, or both. To determine the contribution of proliferation to any observed differences, LCMV-infected mice would be given BrdU in their drinking water during the contraction phase, and the incorporation of this DNA marker into Ag-specific CD8 T cells would be analyzed by flow-cytometric staining with an α -BrdU mAb, in conjunction with D^b/gp33 MHC Class I tetramer, in a method similar to that used by Grayson et al (12).

A decrease in the number of Ag-specific CD8 Thy1.1⁺ T cells in Bmi-1(EC) mice compared to control mice could result from toxicity of Bmi-1 over-expression. However, it is likely that a toxicity

effect would be manifest at the naïve T cell stage or even earlier, since the level of Bmi-1 expression from the constitutive LTR promoter should be the same at all stages of T cell development. Thus far, no difference has been observed in the frequency of Thy1.1⁺ cells in the PBL populations of naïve Bmi-1(EC) and MiT control chimeras. However, it is possible that activated T cells have increased transcription from the proviral LTR and therefore more ectopic Bmi-1. To test this, naïve and activated CD8 T cells would be purified from Bmi-1(EC) chimeras and expression of Bmi-1 in each population determined by quantitative RT-PCR. If Bmi-1 expression is the same in naïve and activated CD8 T cells, yet we observe that only activated CD8 T cells are being adversely affected by ectopic Bmi-1 expression, this would be more consistent with ectopic Bmi-1 specifically promoting the death of CD8 T_E, possibly by pre-maturely activating an apoptotic program in CD8 T_E. This would be tested by analyzing Thy1.1⁺ T cells from Bmi-1(EC) mice at successive time points post-infection for apoptotic markers by Annexin V staining.

C. Direct analysis of anti-apoptotic function of Bmi-1 in activated CD8 T cells.

In interpreting our results thus far, we have assumed that positive or negative effects of Bmi-1 manipulation on CD8 T cell survival were due to an anti or pro-apoptotic function, respectively. Here we directly test whether modulating apoptosis susceptibility is a mechanism of Bmi-1 action in activated CD8 T cells. The anti-apoptotic molecules whose expression is positively regulated by Bmi-1 in murine bone marrow are able to protect cells from both extrinsically and intrinsically-induced apoptosis (41, 54). Therefore, apoptosis will be induced in activated CD8 T cells using both extrinsic and intrinsic triggers—specifically α -CD3 mAb for the former, and cytokine withdrawal for the latter.

Polyclonal splenocytes and lymphocytes will be harvested from B6 mice, activated with α -CD3 + α -CD28, and transduced with MiT, Bmi-1(EC) or Bmi-1(KD) viruses. On day two post-activation, Thy1.1⁺ CD8 T cells from each culture will be positively selected using MACS beads and re-plated separately. For extrinsically-triggered apoptosis, the cells will be cultured an additional two days in the presence of IL-2, and then seeded on wells coated with α -CD3 mAb, which causes apoptosis of activated CD8 T cells in a Fas-dependent, Bcl-2 independent manner (17). To detect apoptotic cells, fluorophore conjugated-Annexin V and 7-actinomycin D (7-AAD) will be used for staining of CD8 T cells, followed by flow-cytometric analysis. AnnexinV is a protein which binds specifically to phosphatidyl serine that is expressed on the surface of early apoptotic cells, and 7-AAD is a vital dye that allows detection of cells with compromised plasma membrane integrity. Aliquots from each culture will be analyzed kinetically to detect cells in both early (AnnexinV⁺7-AAD⁻) and late (AnnexinV⁺ 7-AAD⁺) stages of apoptosis. Specific cell loss on each day will be normalized

to untreated controls, and calculated using the following formula: $Specific\ cell\ loss = 100 \times [1 - (\# \text{ of live cells in treated sample} / \# \text{ of live cells in control sample})]$ (ref).

Alternatively, positively-selected CD8⁺Thy1.1⁺ T cells will be cultured two additional days in the presence of exogenous IL-2. Then, these cells will be washed and cultured in the absence of IL-2 to trigger the intrinsic pathway of apoptosis. Culture of *in vitro*-activated peripheral T cells in the absence of IL-2 causes apoptosis in a Fas-independent manner, and death is inhibited by over-expression of Bcl-2 (17). Cells cultured in the presence of IL-2 will be used as a baseline of comparison for the effects of IL-2 withdrawal, and the optimal concentration of IL-2 which prevents a majority of the activated T cells from apoptotic death will be determined empirically. Cells from each culture will be analyzed at both early and late time-points after IL-2 withdrawal for apoptotic markers as described above.

Possible results and Interpretations

If manipulation of Bmi-1 expression using Bmi-1(EG) and Bmi-1(KD) correlates with an anti-apoptotic role for Bmi-1 in extrinsically-triggered, but not intrinsically-triggered apoptosis, it would suggest that Bmi-1 is important for regulating the numbers of activated T_E *in vivo* under conditions when antigen is still present, since extrinsic death pathways require multiple strong stimulations of the TCR. This hypothesis could be tested under conditions of chronic antigenic stimulation, by infection of Bmi-1(EG) mice with the Armstrong Clone 13 strain of LCMV which causes chronic infection and deletion of CD8 T cell populations specific for immunodominant epitopes (55).

Alternatively, if Bmi-1 only protects CD8 T cells from intrinsic apoptosis, it would suggest that Bmi-1 is important for nascent CD8 T_M survival after pathogen has been cleared, and when cells likely die from cytokine withdrawal. This hypothesis would be tested by harvesting CD8 T_E from day 8 LCMV-infected Bmi-1(EG) chimeras and culturing them *in vitro* with or without γ -chain family cytokines. We expect that Bmi-1 over-expression would relieve the requirement for these cytokines for activated CD8 T cell survival. Similar experiments performed with *bim*^{-/-} CD8 T_E showed that this Bcl family member has a pro-apoptotic role in the death of activated CD8 T cells (32).

If ectopic Bmi-1 protects CD8 T cells from apoptosis by both extrinsic and intrinsic mechanisms, it would suggest that Bmi-1 is a protective factor for activated CD8 T cells at multiple stages of infection. Therefore it is possible that Bmi-1 expression or activity is regulated in concordance with T cell activation. These hypotheses would be tested by quantitative multiplex RT-PCR analysis of Bmi-1, p16, and p19 expression in Ag-specific CD8 T cells harvested from wild-type B6 mice at successive days post-LCMV infection. Interpretations of the results of these experiments would assume that Bmi-1 regulates the same genes in CD8 T cells as it does in other

cell types, however a thorough analysis would require further characterization of Bmi-1 target genes in CD8 T cells.

Limitations and Alternative Approaches

Flow cytometric analysis for quantitation of cell loss has the disadvantage that late apoptotic cells will fragment and leave the standard gate used for acquisition. Therefore, during acquisition of samples, the collection gate will include all events, allowing the numbers of dead cells which have low forward and side scatter values to be included in the analysis. In addition, a time-course analysis will be done beginning 4h after the apoptotic stimulus has been added, to optimize the assay for detection of early apoptotic cells, which will be AnnexinV⁺ 7-AAD⁻.

There are many ways to induce apoptosis, and different lymphocyte populations have been empirically shown to vary in their sensitivity to these different stimuli (56). If we do not observe any difference in the frequency of Bmi-1-manipulated apoptotic T cells compared to control T cells using α -CD3 stimulation and cytokine withdrawal, other methods of apoptosis induction will be used. Specifically, for extrinsic, death-receptor-mediated pathways, direct agonistic stimulation of Fas using an α -Fas Ab cross-linked with a 2^o Ab would be used (25). Additionally, γ -irradiation, serum starvation, and staurosporine are alternative methods to inducing intrinsic apoptotic pathways.

Specific Aim 2: Determine the role of Bmi-1 in the maintenance of Ag-specific CD8

T_M

Rationale:

We hypothesize that Bmi-1 is a positive regulatory factor of CD8 T_M maintenance, since Bmi-1^{-/-} HSCs are unable to self-renew. Specifically, many studies suggest that Bmi-1 controls cell cycle regulatory genes, providing a mechanistic explanation of its self-renewal function in HSCs. We predict that manipulation of Bmi-1 expression in Ag-specific CD8 T_M will result in their increased homeostatic proliferation, manifested as increased cell division and decreased cell death.

It is possible that Bmi-1 is not the limiting factor for proliferation or survival of CD8 T_M, thus over-expression of Bmi-1 would not have an effect on CD8 T_M homeostasis. However, to determine if Bmi-1 has any role in CD8 T_M maintenance, endogenous Bmi-1 expression in a heterogeneous CD8 T_M pool could be analyzed. Specifically, analyses of a CD8 T_M population specific for the gp33 peptide of LCMV have shown that this population is heterogeneous with respect to cell cycle status. The majority of the population is in the G0/G1 phase of the cell cycle, as indicated by 1N DNA content. A small fraction (0.4-1.7%) of the population is in division (S+G2/M), as indicated by 2N DNA content (36, 37). Short 24-hour pulses of BrdU given to LCMV-immune mice showed that the frequency of the dividing T_M population was highest in the bone marrow (~7.0% of gp33-specific

CD8 T_M) and spleen (~2.0 % of gp33-specific CD8 T_M). Since Bmi-1 promotes the cycling of adult HSCs, we hypothesize that Bmi-1 will be expressed at higher levels in cycling CD8 T_M, than non-cycling CD8 T_M.

Experimental Design

Proliferation and survival of Bmi-1-manipulated CD8 T_M. A group of Bmi-1(EC) chimeras or MiT control chimeras will be infected with LCMV Armstrong as described in Aim I. The numbers of CD8 T cells specific for D^b/gp33 in the spleen and LN of each group will be analyzed by tetramer staining on days 8, 15, 30, 45, 60, 90 and 120 post-infection. The memory set-point for the D^b/gp33-specific populations in Bmi-1(EC) and MiT mice will be determined empirically, and comparisons of the number of D^b/gp33⁺ CD8 T cells at subsequent time points will be normalized to these values. To analyze relative proliferation of Bmi-1(EC) and control Ag-specific CD8 T_M populations, LCMV-infected Bmi-1(EC) and MiT chimeras will be fed bromodeoxyuridine (BrdU) in their drinking water seven days prior to each time point indicated above (starting at d15). This thymidine analog is incorporated permanently into the DNA of dividing cells during S phase, and can be detected by mAb staining. Seven days was chosen as a labeling period so that enough cells that have undergone division in the T_M population will be detected to make meaningful comparisons of the frequency of Ag-specific BrdU⁺ cells between groups. Thus, at each time point, the frequency of Ag-specific splenocytes that have incorporated BrdU will be analyzed by MHC D^b/gp33 tetramer and α-BrdU mAb staining, respectively (12, 57).

Endogenous bmi-1 expression in CD8 T_M. When P14 TCR Tg cells are used to generate CD8 T_M, the frequency of total Ag-specific CD8 T_M in these tissues is higher than when non-transgenic pre-cursors are analyzed (36). Therefore, P14-B6 chimeras will be made by adoptive transfer of 1x10⁶ naïve P14 T cells to un-infected, congenic B6 mice. The hosts will then be infected with LCMV Armstrong 24h later. No sooner than 45 days post-infection, bone marrow cells (harvested from the femur and tibia), and splenocytes from LCMV-immune B6-P14 chimeras will be stained with D^b/gp33 tetramer and Hoescht 33342 dye. The latter is a cell permeable DNA binding dye that is incorporated into dividing cells during S phase, thus it can be used during flow cytometric analyses to identify dividing and non-dividing cells in specific lymphocyte populations. Thy1.1 will be used to identify the donor population of P14 T cells. The P14 T_M population will be sorted into cycling and non-cycling cells on the basis of DNA content by FACS. The cell lysates from each population will then be used for quantitative RT-PCR analysis of *bmi-1* expression. Additionally, the abundance of a “housekeeping gene” mRNA and 18S rRNA will be used as internal controls for the efficiency of the RT reaction, and to normalize mRNA input quantity for each sample. SYBR green dye (Molecular Probes) will be used to quantify the amount of *bmi-1* or internal control cDNA

generated by reverse transcription at each amplification cycle. Comparative analyses of *bmi-1* transcript abundance will be based on the cycle threshold (C_t) value for each sample normalized to the internal control abundance.

Possible results and Interpretations

We predict that Bmi-1 over-expression will result in an increase in cell proliferation and a decrease in cell death of Ag-specific CD8 T_M , and thus a net increase in the number of these cells from the end of contraction to the last time point of the analysis. Importantly, we also predict that these results will be observed only in the Thy1.1⁺ CD8 T_M population, and not the Thy1.1⁻ T_M population of the Bmi-1(EC) mice. This result would suggest that Bmi-1 can be a positive regulator of proliferation and/or survival for CD8 T_M . The physiologic relevance of this result will be interpreted along with the analysis of endogenous Bmi-1 expression in CD8 T_M . If Bmi-1 appears to have a role in CD8 T_M maintenance, future work would focus on identifying Bmi-1 target genes using the modified ChiP assays on purified Ag-specific CD8 T_M populations, as discussed in Aim I.

It is possible that there will be fewer Ag-specific CD8 T_M in Bmi-1(EC) mice compared to MiT mice. This could be the result of the rate of cell death exceeding that of cell proliferation, or due to cell death in the absence of cell proliferation. Regardless, decreased numbers would suggest either that Bmi-1 does not promote the proliferation of CD8 T_M , or that over-expressing Bmi-1 is toxic to the CD8 T_M cells. As discussed earlier, the latter possibility is unlikely, due to the fact that Bmi-1 over-expression is tolerated in the progenitors of CD8 T_M , and in other lymphoid lineages. The possibility that Bmi-1 induces the death of CD8 T_M is also unlikely based the published data examining the role of Bmi-1 in other cell types.

Finally, no difference in the rates of cell division and cell death between Bmi-1(EC) and MiT mice would suggest that Bmi-1 does not have a role in the basal homeostatic proliferation of CD8 T_M . However, this result would not disprove our hypothesis, as it is possible that Bmi-1 is not the limiting factor for stimulating proliferation or inhibiting cell death in CD8 T_M , and therefore its over-expression alone would not confer enhanced proliferation or survival on CD8 T_M . As studies of Bmi-1 in other cell types indicate that Bmi-1 mRNA expression directly correlates with its function as a regulator of cell cycle inhibitory genes, we hypothesize that Bmi-1 mRNA expression may correlate with the cell cycle status of CD8 T_M . We predict that Bmi-1 expression will be higher in cells that are in division, which would suggest that Bmi-1 is a positive regulator of the balance of quiescent vs. cycling CD8 T_M cells. To further explore this hypothesis, fetal liver chimeras could be made using the Bmi-1(KD) virus. Infection of these mice with LCMV and subsequent analysis of CD8 T_M populations as described above would address whether Bmi-1 is necessary for maintenance of an Ag-specific CD8 T cell population. In light of the severe hematopoietic defects in *bmi-1*^{-/-} mice, this

experiment could be technically challenging, and an inducible knockout targeted specifically to the CD8 lineage would facilitate this approach.

Limitations and Alternative Approaches

In the Bmi-1(EG) mice, since Bmi-1 is over-expressed at all stages of CD8 T cell development, an increase in the proliferation and/or survival of CD8 T_M may be an indirect effect of enhanced proliferation and/or survival at the CD8 T_N or CD8 T_E stage. However, if Bmi-1 does confer increased homeostatic proliferation on naïve CD8 T cells, one would expect to observe an increase in the number of these cells in Bmi-1(EG) mice compared to control mice. Preliminary data indicates that there is not an increase in the frequency of naïve CD8⁺Thy1.1⁺ T cells in the PBL, LN, or spleen of Bmi-1(EG). And to control for effects on proliferation during contraction, all quantitative analyses of Ag-specific CD8 T_M will be standardized to the number of Ag-specific CD8 T cells at the set point.

The results of the over-expression experiments only indicate if Bmi-1 expression can promote homeostatic proliferation of a CD8 T_M population, but not whether it is necessary for this process. Analysis of homeostatic proliferation in the absence of Bmi-1 expression would directly address this, and the Bmi-1(KD) chimeras were previously discussed. Additionally, CD8 T cells could be activated and transduced with the Bmi-1(KD) virus *ex vivo*, positively sorted for Thy1.1 expression, labeled with the cell division and tracking dye CFSE, and then adoptively transferred into naïve or LCMV-infected mice. Analysis of the cell division and absolute numbers of donor cells in the spleens and LN of host mice at subsequent time points post-transfer would determine if Bmi-1 is necessary for the maintenance of an Ag-specific CD8 T_M population. A similar *in vitro* to *in vivo* transfer system for activated CD8 T cells has been used successfully in end-point analyses of memory CD8 T cell characteristics and could potentially be explored (1,16).

For the RT-PCR analysis of Ag-specific CD8 T_M, it is estimated that 3×10^4 P14 T_M are in cycle in the spleen, and 4×10^4 P14 T_M are in cycle in the bone marrow in LCMV-immune mice (36). Since quantitative RT-PCR analysis can be done on single cells with precision, using kits which are commercially available, we expect to recover enough cells from the cycling Ag-specific CD8 T_M population for these analyses (58).

Use of experimental animals

All experiments described above which require work with animals will be done in accordance with the Standard Operating Procedures of the IACUC of the University of Pennsylvania.

Species. C57/BL6 mice will be purchased from the Jackson Laboratories (Bar Harbor, ME) and housed at the University of Pennsylvania barrier facilities. Animals infected with LCMV Armstrong will be housed in appropriate BSL 2 facilities.

Veterinary Care. The University of Pennsylvania facilities meet all federal and state requirements for animal care. Daily care will be performed by University Laboratory Animal Research personnel and is provided in conformance with NIH standards.

Tail bleeds. Where indicated, LCMV-infected mice will be bled from the tail. A small incision will be made across the lateral tail vein and blood will be collected in capillary tubes. A gauze pad and slight pressure will be applied to the lesion until bleeding has ceased.

Euthanasia. Dissection will be performed after animals have been euthanized, according to the University of Pennsylvania IACUC Guidelines for Euthanasia. Carbon dioxide gas from a compressed cylinder will be infused into a sealed chamber and mice will be observed until respiratory and muscle movements have ceased. To ensure death, cervical dislocation will be performed by trained laboratory personnel.

References

1. S. M. Kaech, R. Ahmed., *Nat. Immunol.* **2**, 415 (2001).
2. A. K. Thomas, C. H. June., *Cancer J.* **7 Suppl 2**, S67 (2001).
3. X. Huang *et al.*, *Blood* **107**, 483 (2006).
4. E. E. Perez, J. L. Riley, R. G. Carroll, D. von Laer and C. H. June., *Clin. Immunol.* **115**, 26 (2005).
5. D. C. Tschärke *et al.*, *J. Exp. Med.* **201**, 95 (2005).
6. S. M. Kaech, E. J. Wherry and R. Ahmed., *Nat. Rev. Immunol.* **2**, 251 (2002).
7. C. J. Luckey *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*(2006).
8. E. J. Wherry *et al.*, *Nat. Immunol.* **4**, 225 (2003).
9. S. M. Kaech, S. Hemby, E. Kersh and R. Ahmed., *Cell* **111**, 837 (2002).
10. A. Cerwenka, T. M. Morgan and R. W. Dutton., *J. Immunol.* **163**, 5535 (1999).
11. K. Murali-Krishna *et al.*, *Immunity* **8**, 177 (1998).
12. J. M. Grayson, L. E. Harrington, J. G. Lanier, E. J. Wherry and R. Ahmed., *J. Immunol.* **169**, 3760 (2002).
13. J. Jacob, D. Baltimore., *Nature* **399**, 593 (1999).
14. J. T. Opferman, B. T. Ober and P. G. Ashton-Rickardt., *Science* **283**, 1745 (1999).
15. N. Manjunath *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **96**, 13932 (1999).
16. N. Manjunath *et al.*, *J. Clin. Invest.* **108**, 871 (2001).
17. A. Strasser, A. W. Harris, D. C. Huang, P. H. Krammer and S. Cory., *EMBO J.* **14**, 6136 (1995).
18. A. T. Vella, S. Dow, T. A. Potter, J. Kappler and P. Marrack., *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3810 (1998).
19. V. S. Marsden, A. Strasser., *Annu. Rev. Immunol.* **21**, 71 (2003).
20. L. Zheng *et al.*, *Nature* **377**, 348 (1995).
21. X. Z. Wang *et al.*, *Immunity* **18**, 631 (2003).
22. M. Lenardo *et al.*, *Annu. Rev. Immunol.* **17**, 221 (1999).
23. J. Dhein, H. Walczak, C. Baumler, K. M. Debatin and P. H. Krammer., *Nature* **373**, 438 (1995).
24. F. Hornung, L. Zheng and M. J. Lenardo., *J. Immunol.* **159**, 3816 (1997).

25. B. Wong, J. Arron and Y. Choi., *J. Exp. Med.* **186**, 1939 (1997).
26. C. Zimmermann, M. Rawiel, C. Blaser, M. Kaufmann and H. Pircher., *Eur. J. Immunol.* **26**, 2903 (1996).
27. L. T. Nguyen *et al.*, *Eur. J. Immunol.* **30**, 683 (2000).
28. A. Reich, H. Korner, J. D. Sedgwick and H. Pircher., *Eur. J. Immunol.* **30**, 678 (2000).
29. A. Reich, H. Korner, J. D. Sedgwick and H. Pircher., *Eur. J. Immunol.* **30**, 678 (2000).
30. P. Bouillet *et al.*, *Science* **286**, 1735 (1999).
31. J. M. Adams, S. Cory., *Science* **281**, 1322 (1998).
32. M. Pellegrini, G. Belz, P. Bouillet and A. Strasser., *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14175 (2003).
33. J. Wan *et al.*, *Immunology* **110**, 10 (2003).
34. F. Petschner *et al.*, *Eur. J. Immunol.* **28**, 560 (1998).
35. D. F. Tough, J. Sprent., *J. Exp. Med.* **179**, 1127 (1994).
36. T. C. Becker, S. M. Coley, E. J. Wherry and R. Ahmed., *J. Immunol.* **174**, 1269 (2005).
37. D. R. Latner, S. M. Kaech and R. Ahmed., *J. Virol.* **78**, 10953 (2004).
38. Y. Haupt, W. S. Alexander, G. Barri, S. P. Klinken and J. M. Adams., *Cell* **65**, 753 (1991).
39. M. van Lohuizen *et al.*, *Cell* **65**, 737 (1991).
40. N. M. van der Lugt *et al.*, *Genes Dev.* **8**, 757 (1994).
41. I. K. Park *et al.*, *Nature* **423**, 302 (2003).
42. A. Iwama *et al.*, *Immunity* **21**, 843 (2004).
43. J. Lessard, G. Sauvageau., *Nature* **423**, 255 (2003).
44. K. Itahana *et al.*, *Mol. Cell. Biol.* **23**, 389 (2003).
45. A. V. Molofsky, S. He, M. Bydon, S. J. Morrison and R. Pardal., *Genes Dev.* **19**, 1432 (2005).
46. K. S. Smith *et al.*, *Mol. Cell* **12**, 393 (2003).
47. J. J. Jacobs, K. Kieboom, S. Marino, R. A. DePinho and M. van Lohuizen., *Nature* **397**, 164 (1999).
48. J. J. Jacobs *et al.*, *Genes Dev.* **13**, 2678 (1999).
49. R. G. Hawley, F. H. Lieu, A. Z. Fong and T. S. Hawley., *Gene Ther.* **1**, 136 (1994).

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50. R. K. Naviaux, E. Costanzi, M. Haas and I. M. Verma., *J. Virol.* **70**, 5701 (1996).
51. H. Pircher, K. Burki, R. Lang, H. Hengartner and R. M. Zinkernagel., *Nature* **342**, 559 (1989).
52. C. A. Schmitt *et al.*, *Cancer. Cell.* **1**, 289 (2002).
53. D. Masopust, V. Vezys, A. L. Marzo and L. Lefrancois., *Science* **291**, 2413 (2001).
54. K. Kuwata *et al.*, *Am. J. Pathol.* **162**, 837 (2003).
55. E. J. Wherry, J. N. Blattman, K. Murali-Krishna, R. van der Most and R. Ahmed., *J. Virol.* **77**, 4911 (2003).
56. R. M. Siegel, Michael J. Lenardo., in *Measurement of apoptosis and other forms of cell death*, John Coligan, Barbara Bierer, David Marguiles, Ethan Shevach and Warren Strober, Eds. (John Wiley & Sons, New York, 2005).
57. K. Murali-Krishna *et al.*, *Science* **286**, 1377 (1999).
58. <http://www.ambion.com/catalog/CatNum.php?1722>