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Impaired memory CD8 T cell responses against an immunodominant retroviral cryptic epitope

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ABSTRACT

The immunodominant cryptic epitope SYNTGRFPPL, encoded within open reading frame 2 of the LP-BM5 retroviral *gag* gene, is critical for protection against retroviral-induced pathogenesis. The goal of this study was to dissect the memory response against this unique immunodominant cryptic epitope. Unlike the protective acute effector population of SYNTGRFPPL-specific CD8 T cells, long-lived SYNTGRFPPL-specific CD8 T cells lacked the ability to protect susceptible mice infected with LP-BM5 retrovirus. Compared to memory CD8 T cells against a conventional epitope with similar MHC-I specificity, primed and restimulated using similar conditions, long-lived SYNTGRFPPL-specific CD8 T cells were impaired in their ability to recall against antigen, with reduced cytolytic capabilities and cytokine production. Since similar priming and restimulation regimes were utilized to generate each effector CD8 T cell population, this study has potentially broad implications with regard to the selection criteria of potent, highly conserved cryptic epitopes for use in epitope-based vaccines.

Introduction

It is well documented that CD8 T cells significantly contribute to the initial control of HIV-1 infection during the acute phase response (Borrow et al., 1994, 1997; Koup et al., 1994), although direct evidence linking superior CD8 T cell immunity with the ability of elite controllers to effectively suppress virus replication is limited (Bailey et al., 2009; Deeks and Walker, 2007; Emu et al., 2008). Recently, it has become apparent that not only the magnitude of the CD8 T cell response but also the qualitative functions of the effector CD8 T cells are important for the control of viral load (Almeida et al., 2007; Critchfield et al., 2008; Ferre et al., 2009). That broad, polyfunctional, vaccine-induced T cell responses are required for the control of heterologous SIV challenge in rhesus macaques (Wilson et al., 2009), and a reduction in the virus set-point of SIV (Liu et al., 2009), indicates the great therapeutic promise of designing T cell-based vaccines to control the genetically diverse circulating isolates and escaped variants of HIV-1 in humans.

Vaccine design has been impeded by the ability of HIV-1 to escape the immune response due to CD8-driven immune pressure (Borrow et al., 1997; Goulder et al., 2001, 1997; Price et al., 1997). Immunization of rhesus macaques with a mosaic polyvalent vaccine containing the sequences of multiple in silico recombined *gag* and *nef* genes resulted in increased T cell recognition of a greater breadth of epitopes from each viral protein and a greater depth of variant sequences of MHC-I-restricted epitopes (Santra et al., 2010). Cryptic epitopes, peptides generated from non-coding sequences or alternative reading frames (ARF), establish an extended source of additional immunogenic peptides for T cell-based vaccines. Immunodominant CD8 T cell responses directed against a cryptic epitope in SIV were consistently detected and correlated to effective control of virus replication in *Mamu-B*17*$^{+}$ elite suppressor rhesus macaques (Maness et al., 2007). Natural in vivo priming against ARF-encoded peptide determinants was also reported to occur during HIV-1 infection in humans (Cardinaud et al., 2004). Importantly, the immunodominant cryptic epitopes were highly conserved among various isolates of the HIV-1 virus, suggesting that preservation of the protein encoded by the primary reading frame is necessary for the fitness and survival of the virus (Cardinaud et al., 2004). Antisense transcripts were also shown to contribute significantly to the pool of immunogenic cryptic epitopes driving virus escape or reversion in a large cohort of South Africans with chronic HIV-1 infection (Bansal et al., 2010), demonstrating the large repertoire of unconventional immunodominant antigens available for vaccines.

Previously, our laboratory has defined a CD8 CTL major, immunodominant, cryptic epitope in a murine model of retrovirus-induced immunodeficiency, namely LP-BM5, which causes a pathology in mice similar to AIDS in humans, termed murine AIDS (MAIDS) (Mayrand et al., 2000, 1998; Schwarz and Green, 1994). The LP-BM5 cryptic epitope, SYNTGRFPPL, is encoded within an ARF, open reading frame

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(ORF) 2, of the retroviral *gag* gene (Mayrand et al., 1998) and was, to our knowledge, the first described ARF epitope in a retroviral system and only the second naturally occurring ARF epitope in any system, the first being in melanomas (Wang et al., 1996; Wang and Rosenberg, 1996). After extensive sequence analysis of the LP-BM5 genome for conventional MHC class I-restricted epitopes, we demonstrated that SYNTGRFPPL was the protective epitope recognized by CD8 T cells during LP-BM5 infection in resistant BALB/c mice (Mayrand et al., 1998; Schwarz and Green, 1994). Using this murine model of retroviral immunodeficiency, our laboratory was the first to establish that CD8 T cells recognizing cryptic epitopes were necessary and sufficient for protection against retroviral pathogenesis: adoptive transfer of SYNTGRFPPL-specific effector CD8 T cells resulted in dramatic, if not complete, protection from disease and viral load when used to reconstitute MAIDS-susceptible $CD8^{-/-}$ BALB/c mice (Ho and Green, 2006b). Due to the similarities between HIV in humans and the disease manifestations of LP-BM5 infection in susceptible mice (Jolicoeur, 1991; Liang et al., 1996; Morse et al., 1992), the MAIDS model of retroviral infection represents an opportunity to study in detail the specific CD8 T cell response, including the memory phase, against cryptic/ARF epitopes encoded by an immunodeficiency causing retrovirus.

The goal of this study was to utilize this unique CTL response to dissect the memory response against an immunodominant cryptic epitope. Here we demonstrate that CD8 T cells recognizing SYNTGRFPPL become phenotypically and functionally distinct over time, compared to CD8 T cells recognizing non-LP-BM5-associated epitopes with similar MHC-I specificity. Specifically, K^d/SYNTGRFPPL-specific CD8 T cells are long lived, have a central memory phenotype, but have a reduced capacity to recall against antigen, as evidenced by a lack of effector cytokines IFNγ, TNFα, and IL-2, and cytolytic capabilities, i.e., these long-lived CD8 T cells exhibit a memory response of poor quality. Although K^d/SYNTGRFPPL-specific CD8 T cells have long-term, antigen-independent persistence when transferred into $CD8^{-/-}$ recipients or congenic recipients with intact CD8 compartments, they fundamentally lack the ability to protect $CD8^{-/-}$ mice infected with LP-BM5 retrovirus, despite their ability to confer protection as acute effector CD8 T cells.

Results

Characterization of the K^d/SYNTGRFPPL-specific CD8 T cell response during protection of BALB/c mice infected with LP-BM5 retrovirus

Acute protection of MAIDS-resistant BALB/c mice infected with murine retrovirus LP-BM5 appears to be solely due to a CD8 T cell response against a single H-$2K^d$-restricted cryptic epitope, SYNTGRFPPL, located within open reading frame 2 of the retroviral *gag* gene (Mayrand et al., 2000, 1998; Schwarz and Green, 1994). Even at high virus doses (2×10^5 pfu, compared to the normal dose of 5×10^4 pfu), LP-BM5-infected BALB/c mice failed to develop disease manifestations typical of murine AIDS (MAIDS), such as severe lymphadenopathy and immunosuppression, measured by spleen weight and lymphocyte responsiveness to mitogens, respectively, measured here at 11 weeks-post infection (Fig. 1A). Although K^d/SYNTGRFPPL-specific CD8 T cells were not readily detectable ex vivo by tetramer staining at early temporal points during infection (data not shown), compared to naive CD8 T cells, SYNTGRFPPL-specific secretion of IFNγ (Fig. 1B), and in vivo lysis of SYNTGRFPPL-pulsed syngeneic target cells, indicated the peak effector response occurred at approximately 10 days post-infection with LP-BM5 (Fig. 1C). This effector CD8 T cell response preceded maximum virus levels, which peaked around day 12 (Fig. 1D). Following expansion of effector CD8 T cells, viral load rapidly declined, so that by days 20–30 of infection, retroviral levels were not readily detected above background (Fig. 1D). As antigen levels diminished, a rapid contraction of the functional CD8 T cell response was observed by 15 days post-infection (Fig. 1B–C).

Characterization of the K^d/SYNTGRFPPL-specific memory CD8 T cell response during protection of susceptible BALB/c $CD8^{-/-}$ mice infected with LP-BM5 retrovirus

Due to the limitations of easily detecting K^d/SYNTGRFPPL-specific tetramer$^+$ CD8 T cells during infection of BALB/c mice with LP-BM5 and in order to investigate the quality of the memory response to SYNTGRFPPL, we utilized an adoptive transfer approach previously developed by our laboratory to confer protection in susceptible BALB/c $CD8^{-/-}$ mice infected with LP-BM5 (Ho and Green, 2006b). These results, coupled with our earlier work, indicated that BALB/c mice are resistant to LP-BM5 pathogenesis due to a protective CD8 T cell response directed against the cryptic epitope SYNTGRFPPL (Mayrand et al., 2000, 1998; Schwarz and Green, 1994). Thus, this adoptive transfer approach adequately recapitulated the CD8-mediated protection observed in intact BALB/c mice, presenting a unique opportunity to study this protective CD8 T cell response in detail.

Acute effector CD8 T cells were generated by priming BALB/c mice with Vac-DG, a recombinant vaccinia virus with the *gag* gene of the LP-BM5 retrovirus inserted (Schwarz and Green, 1994). Three weeks post-priming, splenocytes were harvested from the primed BALB/c mice, and K^d/SYNTGRFPPL-specific effector CD8 T cells were cultured in vitro for 9 days with synthetic SYNTGRFPPL peptide and low dose IL-2. Prior to transfer into $CD8^{-/-}$ mice, as we have previously published (Ho and Green, 2006b), the K^d/SYNTGRFPPL-specific effector population consisted of approximately 30% CD8 T cells specific for SYNTGRFPPL based upon tetramer staining and functional analysis. CTL function was robust: antigen-specific lysis of chromium-labeled target cells pulsed with SYNTGRFPPL peptide was significantly greater than lysis of target cells pulsed with irrelevant GFNKLRSTL peptide ($P<0.01$) (Supplementary Fig. 1A). Substantial SYNTGRFPPL-specific co-production of IFNγ and TNFα was also observed after in vitro restimulation with SYNTGRFPPL peptide (36.2% versus 3.3% when restimulated with irrelevant GFNKLRSTL peptide), along with a minor population of CD8 T cells producing IFNγ and TNFα and co-producing IL-2 after restimulation with SYNTGRFPPL peptide (0.99% versus 0% when restimulated with irrelevant GFNKLRSTL peptide) (Supplementary Fig. 1B). Based on tetramer analysis of the effector CD8 T cells, expression of CD62L and CD127 (IL-7Rα) were roughly similar to the expression of naive CD8 T cells (Supplementary Fig. 1C), while surface expression of the activated isoform of CD43 (1B11), CD44, and CD122 (IL-2Rβ) were all high, compared to naive CD8 T cells.

Eleven weeks post-transfer of the acute K^d/SYNTGRFPPL-specific effector CD8 T cells into $CD8^{-/-}$ mice, we were able to detect equivalent percentages and numbers of long-lived K^d/SYNTGRFPPL-specific CD8 T cells by tetramer analysis of lymphocytes isolated from the spleens of both LP-BM5-infected and non-infected $CD8^{-/-}$ recipient mice (Fig. 2A), indicating that maintenance of the transferred CD8 T cells was antigen-independent. Phenotypically, when the CD62L and CD122 expression was compared between long-lived and acute effector K^d/SYNTGRFPPL-specific CD8 T cells, there was roughly similar expression by each antigen-specific population. In particular, when the MFI of CD122 and CD62L were normalized to the expression on naive control CD8 T cells, long-lived and acute effector K^d/SYNTGRFPPL-specific CD8 T cells had a comparatively similar fold-change in expression of these two proteins (Fig. 2B, Supplementary Fig. 2). However, compared to acute effector CTL, the expression of CD127 and CD44 on long-lived memory K^d/SYNTGRFPPL-specific CD8 T cells showed a trend towards being approximately 50–60% higher when normalized to the expression on naive CD8 T cells, although the increases in CD127 and CD44 levels were not statistically significant compared to the expression on acute effector CTL (Supplementary Fig. 2).

Unlike acute SYNTGRFPPL-specific effector CD8 T cells, functional defects were observed in the long-lived SYNTGRFPPL-specific CD8 T cell population after restimulation with SYNTGRFPPL peptide, as there were minimal, if any, cells within the transferred population able to produce

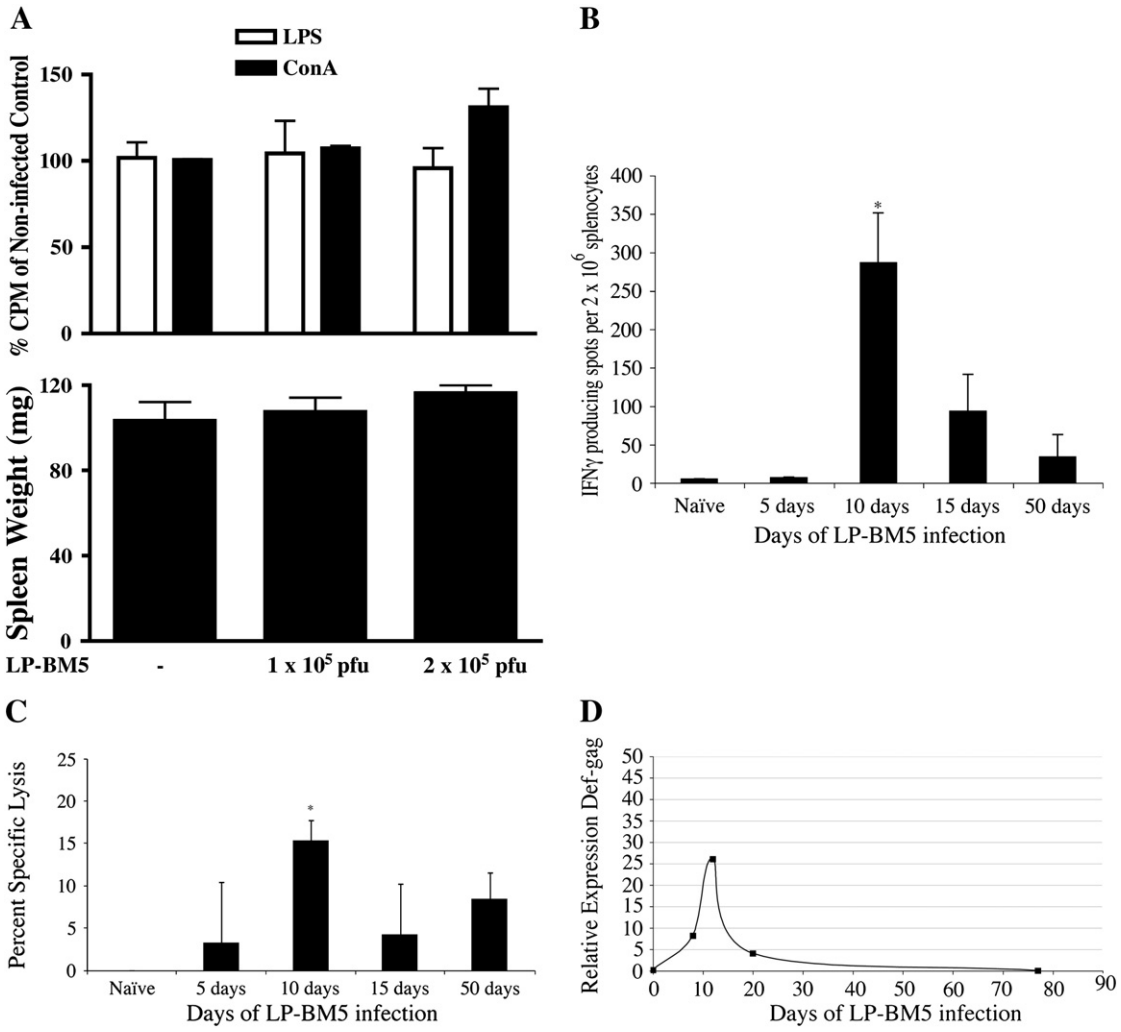


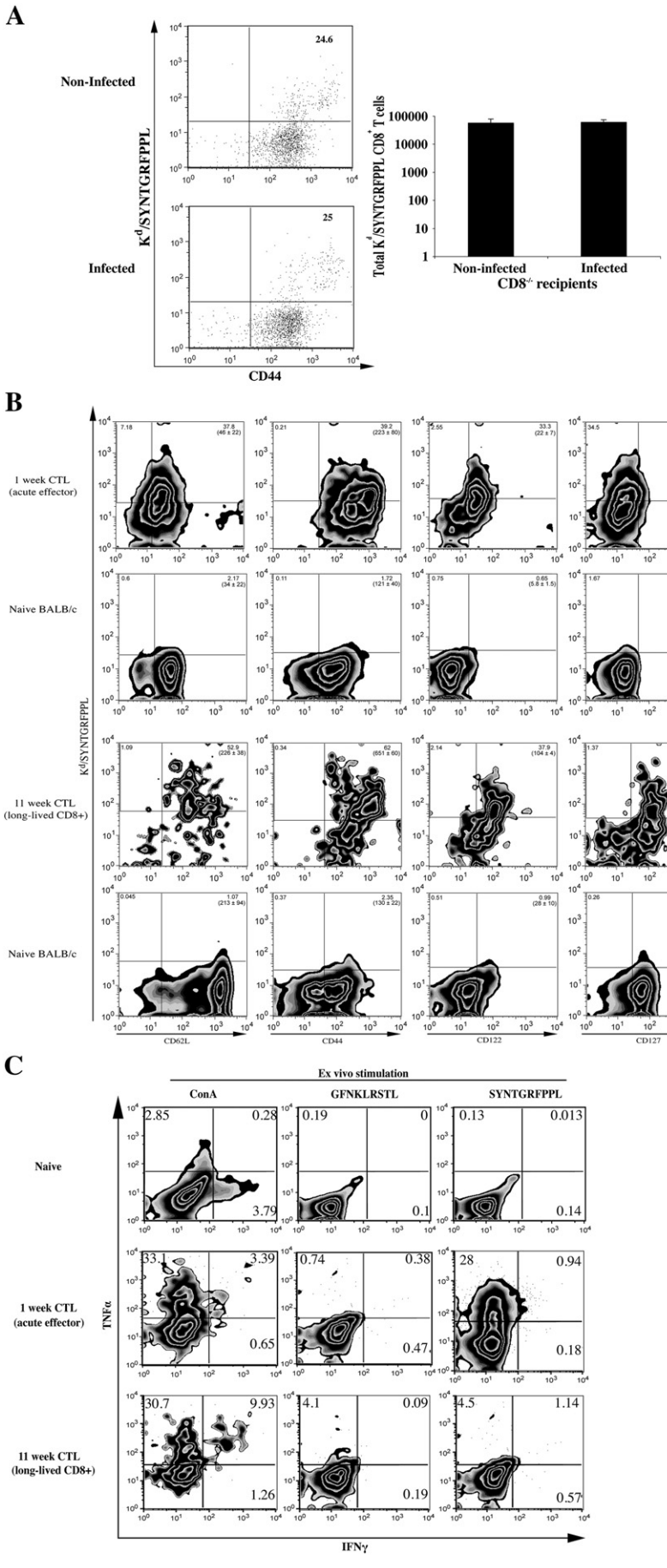
Fig. 1. Characterization of the K^d/SYNTGRFPPL-specific effector and memory CD8 T cell response during LP-BM5 infection of BALB/c mice. (A) BALB/c mice were infected with 1 or 2×10^5 pfu of LP-BM5 retrovirus for 11 weeks, after which disease was assessed. The bottom panel, spleen weight; top panel, B and T cell responsiveness to mitogen stimulation with LPS and ConA, respectively. The data are represented as the percentage counts per minute (CPM) relative to the appropriate non-infected control. (B–C) Functional characterization of the K^d/SYNTGRFPPL-specific CD8 T cell response during LP-BM5 infection in BALB/c mice. (B) ELISPOT analysis of IFNγ production by effector and memory CTL. Splenocytes were isolated from BALB/c mice infected for 5, 10, 15, or 50 days with LP-BM5 retrovirus and stimulated ex vivo for 72 h with SYNTGRFPPL peptide. $^*P<0.05$. (C) In vivo cytolysis of SYNTGRFPPL-pulsed syngeneic target splenocytes in BALB/c mice infected with LP-BM5 for 5, 10, 15, or 50 days at the time of target cell injection. Significance is in comparison to control naive BALB/c mice. $^*P<0.05$. (D) Viral RNA expression of BM5def from spleen-derived RNA samples isolated from LP-BM5-infected BALB/c mice at the indicated temporal points. Expression levels of BM5def are relative to the cycle threshold values obtained for β-actin controls.

effector cytokines IFNγ and TNFα during an antigen-specific recall response, compared to the higher proportion (28%) of the acute effector CTL population able to produce TNFα (Fig. 2C). When stimulated with ConA, approximately 35–40% of the remaining transferred CD8 T cells were able to produce TNFα and/or IFNγ (Fig. 2C). These results indicated that, although long-lived SYNTGRFPPL-specific CD8 T cells had phenotypic attributes similar to canonical central memory T cells as described in the literature (Wherry et al., 2003) ($CD44^{high}$ and $CD62L^{moderate/high}$), they appeared to have functional deficiencies when restimulated with peptide ex vivo.

Impaired recall capacity and memory responsiveness are unique to long-lived K^d/SYNTGRFPPL-specific CD8 T cells

To confirm whether the long-lived K^d/SYNTGRFPPL-specific CD8 T cells had severely diminished function and to demonstrate that any

Fig. 2. Eleven weeks post-transfer into $CD8^{-/-}$ recipient mice, K^d/SYNTGRFPPL-specific CD8 T cells are long-lived and are unable to produce cytokines in response to peptide restimulation. (A) Percentages and total numbers of K^d/SYNTGRFPPL-specific CD8 T cells remaining 11 weeks post-transfer into either naive or LP-BM5-infected $CD8^{-/-}$ recipient mice. Plots are gated on total $CD8^+$ lymphocytes. Numbers in upper right quadrants indicate percent of tetramer-positive K^d/SYNTGRFPPL-specific CD8 T cells. In the right-hand panel, total numbers of tetramer-positive K^d/SYNTGRFPPL-specific CD8 T cells are calculated, based upon total splenic leukocytes. (B) Surface staining of CD62L, CD44, CD122, and CD127 expression on K^d/SYNTGRFPPL-specific $CD8^+$ splenocytes isolated either 1 week post-transfer into non-infected $CD8^{-/-}$ recipients (to characterize naive versus acute effector $CD8^+$ splenocytes at an early effector stage) or 11 weeks post-transfer (to characterize naive versus long-lived SYNTGRFPPL-specific $CD8^+$ splenocytes at a memory stage). Plots are gated on $CD8^+$ cells. Numbers in the upper quadrants represent the percentage of total $CD8^+$ T cells staining for each marker. Numbers in parentheses indicate the MFI ± standard deviation for each protein expressed on total $CD8^+$ tetramer-specific T cells; for the naive controls, MFI is calculated based on total $CD8^+$ T cells. (C) Antigen-specific IFNγ and TNFα cytokine production after stimulation for 24 h ex vivo with synthetic peptide. Plots represent intracellular staining of naive, acute effector, or long-lived $CD8^+$ T cells stimulated ex vivo for 24 h with synthetic SYNTGRFPPL peptide + IL-2 or irrelevant GFNKLRSTL peptide + IL-2. Stimulation with ConA was used as a positive indicator of IFNγ and TNFα cytokine production. Plots are gated on $CD8^+$ cells. Numbers in quadrants represent percentage of total $CD8^+$ T cells staining positive for each cytokine. Data are representative of at least 2 experiments with 4–6 animals per group.


A
Non-Infected
Infected
24.6
25
Kd/SYNTGRFPPL
CD44
Total Kd/SYNTGRFPPL CD8+ T cells
Non-infected
Infected
CD8-/- recipients
B
1 week CTL (acute effector)
Naive BALB/c
11 week CTL (long-lived CD8+)
Naive BALB/c
Kd/SYNTGRFPPL
CD62L
CD44
CD122
CD127
C
Ex vivo stimulation
ConA
GFNKLRSTL
SYNTGRFPPL
Naive
1 week CTL (acute effector)
11 week CTL (long-lived CD8+)
TNFα
IFNγ

differences in function were not simply due to the adoptive transfer system, we compared effector function of transferred K^d/SYNTGRFPPL versus K^d-restricted gammaherpesvirus-specific, long-lived effectors, the latter of which were elicited with an identical stimulation protocol. Thus, BALB/c mice were primed with either Vac-DG or Vac-M2, a recombinant vaccinia virus containing the latent gammaherpesvirus gene, for 3 weeks followed by further in vitro expansion with either synthetic peptide SYNTGRFPPL or GFNKLRSTL, the immunodominant H-2K^d-restricted gammaherpesvirus latent peptide, for 9 days. Following culture, $CD8^{-/-}$ recipient mice were reconstituted with equal ratios of effector CD8 T cells specific for each epitope, SYNTGRFPPL and GFNKLRSTL, based upon tetramer analysis.

Prior to transfer of these two acute effector populations, clear functional and phenotypic differences between each population were observed. As expected, after 9 days of in vitro restimulation with GFNKLRSTL peptide, acute K^d/GFNKLRSTL-specific effector CD8 T cells had a classic effector phenotype in which a majority (>60%) of antigen-specific CD8 T cells were $CD62L^{low}$ and essentially all $tetramer^+$ cells were $CD44^{high}$ (Fig. 3A). However, although analogously generated K^d/SYNTGRFPPL-specific acute effector CD8 T cells were capable of antigen-specific lysis and cytokine production (Fig. 3B–C), they retained significantly higher expression levels of CD62L ($P<0.05$) compared to GFNKLRSTL-primed CTL, a phenotype similar to either naive or resting central memory CD8 T cells (Fig. 3A, Supplementary Fig. 1C). Reflective of this phenotypic difference in CD62L expression between the two effector populations, cytokine production also differed. Co-production of the effector cytokines IFNγ and TNFα by GFNKLRSTL-primed effector CD8 T cells was observed only in the $CD62L^{low}$ population (Fig. 3B) after restimulation with GFNKLRSTL peptide, phenotypically characteristic of effector CD8 T cells. In sharp contrast, it was the large proportion of $CD62L^{moderate/high}$ SYNTGRFPPL-primed CD8 T cells that produced both IFNγ and TNFα in response to homologous peptide restimulation (Fig. 3B). There were essentially no cells producing effector cytokines within the $CD62L^{low}$ population of SYNTGRFPPL-primed CD8 T cells after restimulation with SYNTGRFPPL.

Although these two acute effector populations had differing expression patterns of CD62L with correspondingly differing production of effector cytokines between the $CD62L^{low}$ vs. $CD62L^{moderate/high}$ populations, they exhibited equivalent in vitro cytolysis of relevant antigen-pulsed target cells (Fig. 3C). The ability of acute K^d/SYNTGRFPPL-specific effector CD8 T cells to lyse SYNTGRFPPL-pulsed target cells was expected, as it has been previously reported that they protect susceptible $CD8^{-/-}$ mice infected with the LP-BM5 retrovirus through a perforin-mediated mechanism (Rutkowski et al., 2009). Collectively, these data indicate that the unique effector phenotype of acute K^d/SYNTGRFPPL-specific effector CD8 T cells is not a result of our experimental system of in vitro restimulation, as acute K^d/GFNKLRSTL-specific effector CD8 T cells, which were generated under identical conditions, are phenotypically and functionally similar to classic acute effector CD8 T cells.

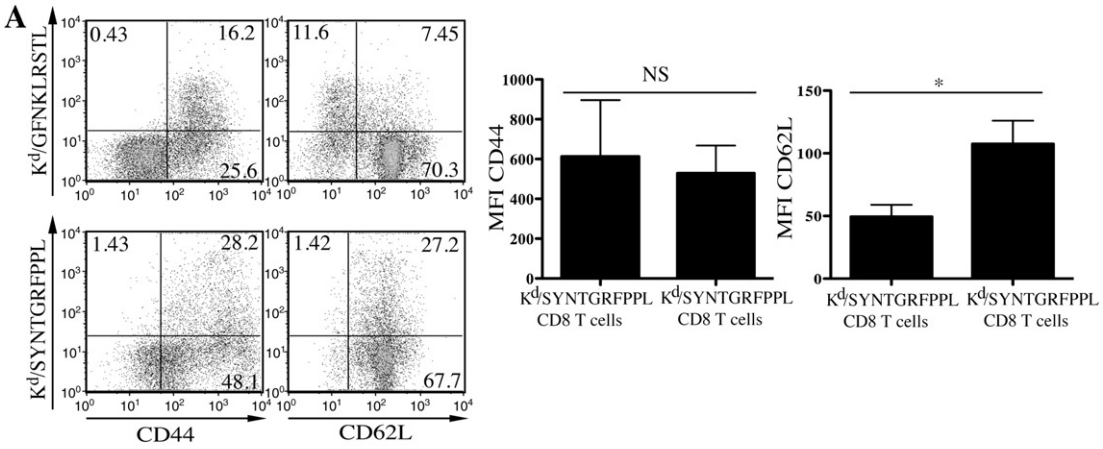
Equal numbers of such acute K^d/SYNTGRFPPL-specific and K^d/GFNKLRSTL-specific effector CD8 T cells were then transferred either into individual $CD8^{-/-}$ recipient mice, or at a 1:1 ratio in the same $CD8^{-/-}$ recipient mouse. Eleven weeks post-transfer, the ability of each long-lived effector population to recall was compared by measuring ex vivo cytokine production and in vivo antigen-specific lysis. Approximately equivalent numbers of each effector population were detectable by tetramer analysis at this temporal point (data not shown), to eliminate the possibility that any observed differences in function were due to the transfer or survival of greater numbers of tetramer-specific T cells in either population.

It was possible that, in comparison to the immunodominant vaccinia-specific memory effector population also present within mice receiving acute K^d/SYNTGRFPPL-specific effector CD8 T cells, the lack of full function in the long-lived K^d/SYNTGRFPPL-specific population was due to a proportionately minimal acute lytic response. If this were the case, after the contraction phase, detection of memory ex vivo would be difficult without expansion of long-lived K^d/SYNTGRFPPL-specific CD8 T cells in vitro. To test this, BALB/c mice were primed with Vac-DG, and after 5 days, splenic CD8 T cells from the primed mice were isolated and used to reconstitute $CD8^{-/-}$ mice. After a 3-day rest period, recipient mice were injected with a 1:1 ratio of CFSE-labeled syngenic target cells pulsed with SYNTGRFPPL- or the Vac K^d-presented epitope, KYGRLFNEI, to monitor the in vivo cytolytic capabilities of the corresponding two specific CD8 T cell effector populations. After 16 h, compared to non-pulsed control targets, lysis of each target population was roughly equivalent, demonstrating that as acute effectors, both K^d/SYNTGRFPPL-specific and K^d/KYGRLFNEI-specific CD8 T cells were able to lyse relevant peptide-pulsed target cells 7–8 days post-priming in vivo (Fig. 4A). Thus, the altered cell-surface phenotype and diminished cytokine production of long-lived SYNTGRFPPL-specific CD8 T cells are more than likely not a result of a proportionately diminished initial acute effector response, at least in terms of cytolytic capabilities.

In the $CD8^{-/-}$ recipients receiving equivalent numbers of each acute effector population (detected by tetramer staining), significantly greater percentages of CD8 T cells were able to co-produce effector cytokines IFNγ and TNFα after restimulation with GFNKLRSTL peptide compared to CD8 T cells recalled with SYNTGRFPPL peptide (8.05% versus 2.49%, $P<0.01$) (Fig. 4B). The relative inability of CD8 T cells to produce effector cytokines in response to SYNTGRFPPL restimulation ex vivo was also observed in $CD8^{-/-}$ recipient mice receiving only acute K^d/SYNTGRFPPL-specific effector CD8 T cells compared to the cytokine response of the same T cells after restimulation with irrelevant GFNKLRSTL peptide (1.74% versus 2.96%) (Fig. 4B). Conversely, GFNKLRSTL-specific cytokine production observed from CD8 T cells isolated from $CD8^{-/-}$ mice receiving only acute K^d/GFNKLRSTL-specific effector CD8 T cells was significantly greater compared to cytokine production of the same group of T cells restimulated with irrelevant SYNTGRFPPL peptide (5.49% versus 1.42%, $P<0.01$) (Fig. 4B). Thus, the defect in cytokine production of long-lived K^d/SYNTGRFPPL-specific CD8 T cells collected from mice receiving equivalent numbers of each effector population 11 weeks prior was not due to a competitive/fitness advantage that the K^d/GFNKLRSTL-specific population may have had over K^d/SYNTGRFPPL-specific CD8 T cells after transfer into the same host.

Since, in each epitope-specific system, $CD8^{-/-}$ mice received a heterogeneous population of CD8 T cells that were generated through initial priming with analogous vaccinia virus vectors, we also compared the ability of vaccinia-specific CD8 T cells collected from mice receiving acute K^d/SYNTGRFPPL- or K^d/GFNKLRSTL-specific CD8 T cells, or in mice administered equal proportions of acute K^d/SYNTGRFPPL- and K^d/GFNKLRSTL -specific CD8 T cells, to recall upon stimulation with one of the immunodominant H-2K^d vaccinia epitopes, KYGRLFNEI (Oseroff et al., 2008; Tscharke et al., 2006). Compared to the proportions of CD8 T cells co-producing IFNγ and TNFα when restimulated with irrelevant SYNTGRFPPL peptide, there was a slightly greater (although not significant) proportion of CD8 T

Fig. 3. Acute effector K^d/SYNTGRFPPL ORF2-specific CD8 T cells have a unique phenotype but similar functional capabilities compared to acute effector CD8 T cells recognizing a conventional antigen after priming and stimulation under similar conditions. Effector CTLs were derived from BALB/c mice primed for 3 weeks with either Vac-DG or Vac-M2, followed by in vitro stimulation for an additional 9 days with SYNTGRFPPL or GFNKLRSTL peptide, respectively. (A) Phenotypic comparison of CD62L and CD44 surface staining on effector K^d/SYNTGRFPPL- or K^d/GFNKLRSTL-specific CD8 T cells. Plots represent gating on tetramer-specific $CD8^+$ cells. The bottom panels represent the MFI for CD44 and CD62L expression on K^d/SYNTGRFPPL- or K^d/GFNKLRSTL tetramer-specific CD8 T cells. (B) Intracellular expression of IFNγ and TNFα by $CD62L^{low}$ and $CD62L^{high}$ effector $CD8^+$ T cells stimulated with SYNTGRFPPL or GFNKLRSTL peptides. Intracellular plots are gated on $CD62L^{high}$ or $CD62L^{low}$ $CD8^+$ lymphocytes. (C) Peptide-specific lysis of SYNTGRFPPL- or GFNKLRSTL-pulsed target cells labeled with ^{51}Cr by K^d/SYNTGRFPPL- or K^d/GFNKLRSTL-specific CD8 T cells.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0.43
16.2
25.6
11.6
7.45
70.3
Kd/SYNTGRFPPL
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48.1
1.42
27.2
67.7
CD44
CD62L
NS
MFI CD44
Kd/SYNTGRFPPL CD8 T cells
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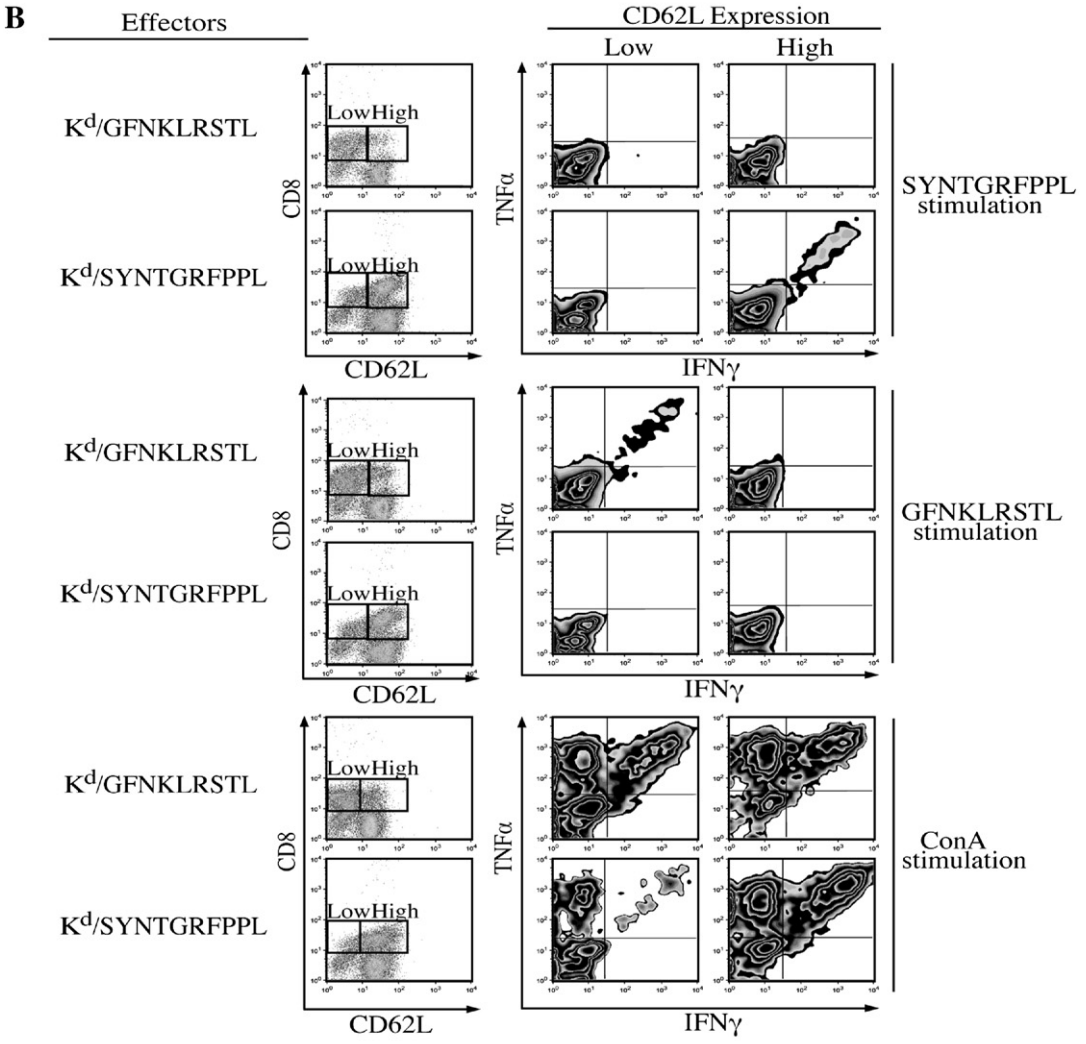
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Effectors
CD62L Expression
Low
High
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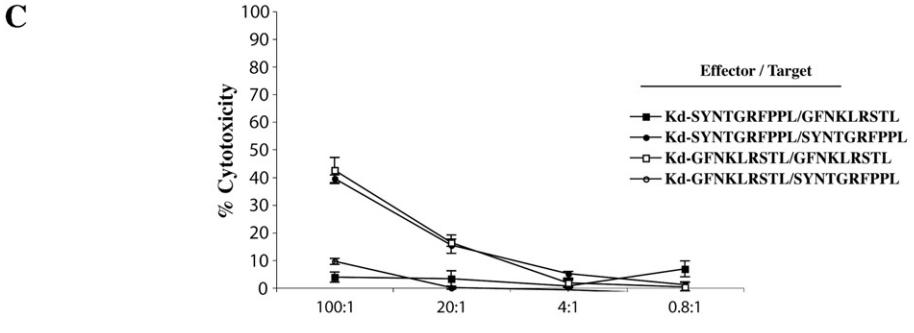
C
% Cytotoxicity
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Kd-SYNTGRFPPL/GFNKLRSTL
Kd-SYNTGRFPPL/SYNTGRFPPL
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100:1
20:1
4:1
0.8:1

cells co-producing IFNγ and TNFα after stimulation ex vivo with KYGRLFNEI peptide in mice receiving adoptive transfer of acute K^d/SYNTGRFPPL-specific CD8 T cells (4.73% versus 1.74%) (Fig. 4B). In $CD8^{-/-}$ mice receiving acute K^d/GFNKLRSTL-specific CD8 T cells, there was a slightly lower percentage of CD8 T cells co-producing IFNγ and TNFα upon restimulation with KYGRLFNEI peptide compared to GFNKLRSTL-responsive T cells (2.26% versus 5.49%) (Fig. 4B). However, the percentage of CD8 T cells identified from recipient mice receiving equivalent numbers of both acute K^d/GFNKLRSTL- and K^d/SYNTGRFPPL-specific CD8 T cells that co-produced IFNγ and TNFα when stimulated with KYGRLFNEI was significantly greater than the proportion of CD8 T cells co-producing effector cytokines IFNγ and TNFα upon restimulation with SYNTGRFPPL peptide (5.11% versus 2.49%, $P<0.01$) (Fig. 4B). Collectively, these results indicated that K^d/KYGRLFNEI- and K^d/GFNKLRSTL-specific CD8 T cell memory cytokine responses raised in parallel to H-2K^d-restricted epitopes are of a qualitatively greater magnitude than the memory cytokine response to the cryptic epitope SYNTGRFPPL.

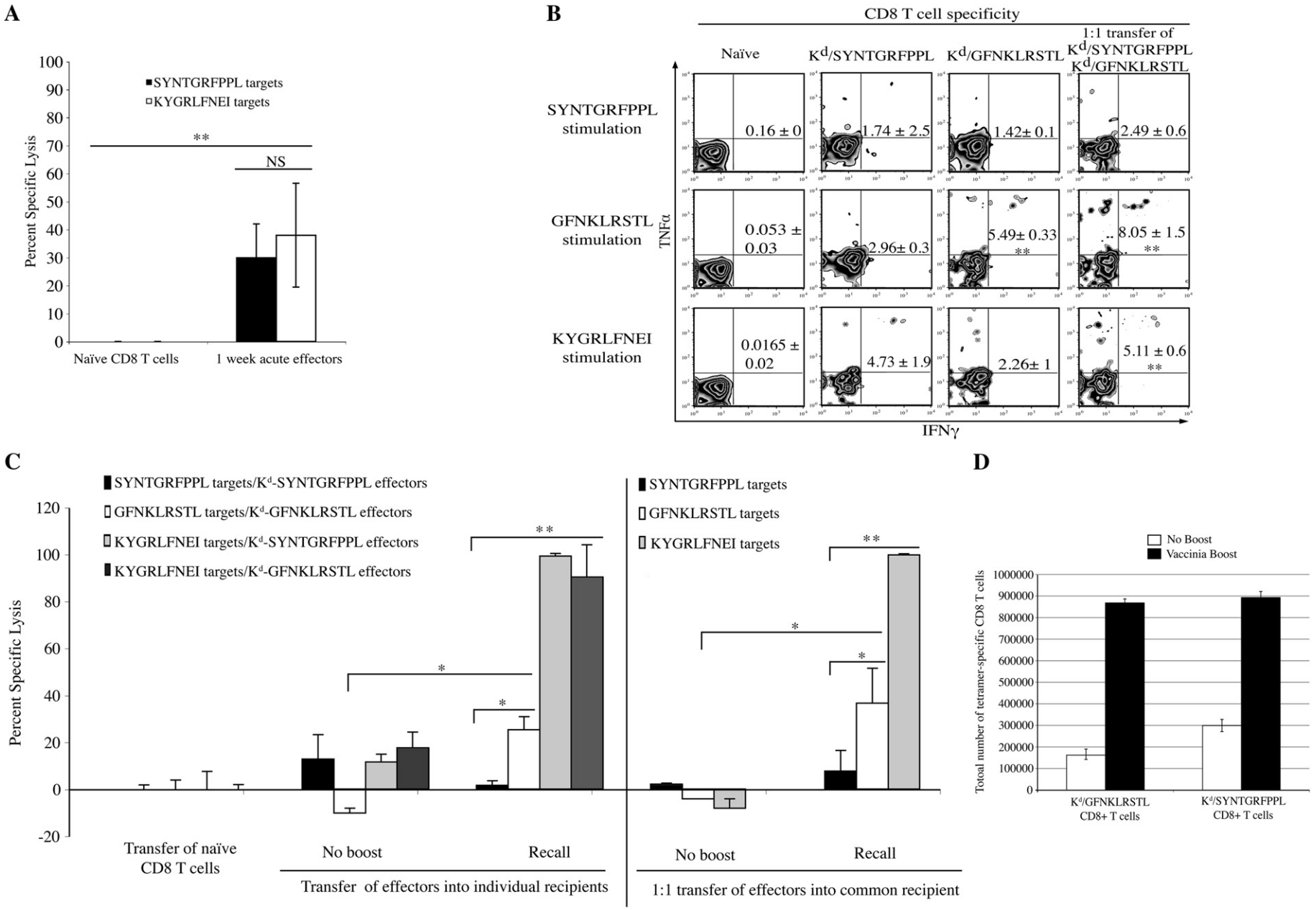
Acute K^d/SYNTGRFPPL-specific effector CD8 T cells utilize perforin-mediated cytolysis of LP-BM5-infected cells as the preferential effector mechanism during protection of LP-BM5-infected $CD8^{-/-}$ mice, as previously reported (Rutkowski et al., 2009). Therefore, the in vivo cytolytic ability of memory CD8 T cells during recall in this co-adoptive transfer model was tested. We measured lysis by immunizing $CD8^{-/-}$ recipient mice that were previously reconstituted with either acute K^d/SYNTGRFPPL-specific or K^d/GFNKLRSTL-specific effector CD8 T cells, followed by administration of either Vac-DG or Vac-M2, to boost the long-lived K^d/SYNTGRFPPL-specific or K^d/GFNKLRSTL-specific memory CD8 T cells, respectively. Boosting was followed by challenge with two target cell types delivered, in each case, to individual recipient mice: either a 1:1 ratio of SYNTGRFPPL- and KYGRLFNEI-, or GFNKLRSTL- and KYGRLFNEI-, pulsed fluorescently labeled syngeneic targets to mice reconstituted with acute K^d/SYNTGRFPPL-specific or K^d/GFNKLRSTL-specific effector CD8 T cells, respectively. After boosting with Vac-DG or Vac-M2 in recipients initially receiving single populations of either acute K^d/SYNTGRFPPL- or K^d/GFNKLRSTL-specific effector CD8 T cells (i.e. 11 weeks prior), significantly greater lysis of both GFNKLRSTL- and KYGRLFNEI-pulsed targets was observed, compared to the lysis of SYNTGRFPPL-pulsed targets ($P<0.05$ and $P<0.01$, respectively) (Fig. 4C, left panel). Defective lysis of SYNTGRFPPL-pulsed targets was also observed after boosting with vaccinia vectors in recipients receiving a 1:1 ratio of each acute effector population (Fig. 4C, right panel). The recall response of long-lived K^d/GFNKLRSTL-specific CD8 T cells after boosting withVac-M2 was significantly greater than the primary response, prior to boosting with Vac-M2, both in recipients receiving single populations of CD8 T cells, or in recipients receiving a 1:1 ratio of acute K^d/SYNTGRFPPL- and K^d/GFNKLRSTL-specific effector CD8 T cells ($P<0.05$) (Fig. 4C). Conversely, lysis of SYNTGRFPPL-pulsed target cells was undetectable/low and essentially unchanged after Vac-DG recall (Fig. 4C). The near inability of Vac-DG-recalled SYNTGRFPPL-specific memory cells to lyse targets was not due to diminished cell numbers, as a comparable increase in the total number of K^d/SYNTGRFPPL- and K^d/GFNKLRSTL-specific CD8 T cells was observed after rechallenge with Vac-DG or Vac-M2, respectively (Fig. 4D). These data, in combination with the cytokine production data in Fig. 4B, indicate that on a per-cell basis, compared to the memory response of both K^d/GFNKLRSTL- and K^d/KYGRLFNEI-specific memory CD8 T cells, long-lived K^d/SYNTGRFPPL-specific CD8 T cells have a memory response of much lower quality. Although long-lived K^d/SYNTGRFPPL-specific CD8 T cells are maintained in response to antigen, they very poorly produce effector cytokines or lyse peptide-pulsed target cells after restimulation with a vaccinia vector.

Long-lived K^d/SYNTGRFPPL-specific CD8 T cells are unable to confer protection in $CD8^{-/-}$ recipient mice upon challenge with LP-BM5 retrovirus

Since long-lived K^d/SYNTGRFPPL-specific CD8 T cells had a memory response of poor quality, we reasoned that these long-lived CD8 T cells might be unable to confer protection in $CD8^{-/-}$ mice during infection with LP-BM5 retrovirus, despite the fact that K^d/SYNTGRFPPL-specific CD8 T cells were protective as acute effectors. To test this hypothesis, we utilized two different approaches: (1) direct LP-BM5 infection of naive (not initially infected) $CD8^{-/-}$ recipient mice previously administered acute effector K^d/SYNTGRFPPL-specific CD8 T cells 11 weeks prior and (2) recovery of long-lived K^d/SYNTGRFPPL-specific CD8 T cells either from LP-BM5-infected primary $CD8^{-/-}$ recipient mice that were protected during initial LP-BM5 infection (antigen-experienced K^d/SYNTGRFPPL-specific CD8 T cells) or from non-infected (antigen-independent K^d/SYNTGRFPPL-specific CD8 T cells) primary $CD8^{-/-}$ recipient mice given acute K^d/SYNTGRFPPL-specific CD8 T cells 11 weeks prior. In approach 2, long-lived K^d/SYNTGRFPPL-specific CD8 T cells were recovered from these primary $CD8^{-/-}$ recipient mice and transferred into secondary LP-BM5-infected $CD8^{-/-}$ recipients. As we have previously published (Ho and Green, 2006b), the transfer of acute K^d/SYNTGRFPPL-specific effector CD8 T cells into primary $CD8^{-/-}$ recipient mice resulted in significant protection compared to LP-BM5-infected $CD8^{-/-}$ recipients that did not receive transfer of CD8 T cells, as evidenced by all disease readouts and viral load (Supplementary Fig. 3A–D) and as summarized by disease index (Fig. 5A).

Although acute K^d/SYNTGRFPPL-specific effector CD8 T cells were able to protect against LP-BM5-mediated disease in the primary $CD8^{-/-}$ recipients (Fig. 5A), long-lived K^d/SYNTGRFPPL-specific CD8 T cells isolated from primary $CD8^{-/-}$ recipients 11 weeks post-transfer were unable to confer equivalent protection compared to the disease index of non-infected control $CD8^{-/-}$ mice depicted in Fig. 5D, regardless of whether they were antigen-independent and negatively purified from non-infected primary $CD8^{-/-}$ recipients receiving acute K^d/SYNTGRFPPL-specific effector CD8 T cells (Fig. 5B, left histogram, and Supplementary Fig. 4) or experienced antigen and were negatively purified from primary $CD8^{-/-}$ recipients previously infected with LP-BM5 (Fig. 5C and Supplementary Fig. 4). Diminished protection was not merely a result of the system of purification and transfer of long-lived K^d/SYNTGRFPPL-specific CD8 T cells, as direct infection of primary $CD8^{-/-}$ recipient mice given acute effector CTL 11 weeks prior also developed significant disease (Fig. 5B, right histogram, and Supplementary Fig. 4). These data indicate that, although acute K^d/SYNTGRFPPL-specific effector CD8 T cells are able to confer protection during LP-BM5 infection in primary $CD8^{-/-}$ recipients, their

Fig. 4. Compared to K^d/GFNKLRSTL-specific memory and recalled CD8 T cells, long-lived K^d/SYNTGRFPPL-specific CD8 T cells have impaired functional memory/recall responses. Effector CTLs, derived from either Vac-DG or Vac-M2 primed BALB/c mice, were either transferred into individual $CD8^{-/-}$ recipient mice or co-transferred into a recipient $CD8^{-/-}$ mouse at a 1:1 ratio based upon tetramer staining. (A) Acute 5-day Vac-DG primed effector CD8 T cells recognizing SYNTGRFPPL or KYGRLFNEI have similar cytolytic capabilities after transfer into $CD8^{-/-}$ recipients. In vivo cytolysis was measured by transferring LP-BM5-specific SYNTGRFPPL- and vaccinia-specific KYGRLFNEI-pulsed target splenocytes labeled with different ratios of CFSE into $CD8^{-/-}$ recipients. (B) Intracellular staining of IFNγ and TNFα by long-lived K^d/SYNTGRFPPL- or K^d/GFNKLRSTL-specific $CD8^+$ T cells 11 weeks post-transfer into individual $CD8^{-/-}$ recipients or into the same $CD8^{-/-}$ recipient at a 1:1 ratio. Numbers in quadrants represent percent mean ± standard deviation of total $CD8^+$ T cells staining positive for each cytokine. (C) In vivo cytolysis of SYNTGRFPPL-, KYGRLFNEI-, or GFNKLRSTL-pulsed target cells by long-lived memory (no boost) and Vac-DG boosted K^d/SYNTGRFPPL-, or Vac-M2 boosted K^d/GFNKLRSTL-, specific CD8 T cells 11 weeks post-transfer into individual $CD8^{-/-}$ recipients, or co-transferred into the same recipient at a 1:1 ratio based upon tetramer staining. (D) Increased numbers of long-lived K^d/SYNTGRFPPL- or K^d/GFNKLRSTL-specific $CD8^+$ T cells 5 days post-boosting with vaccinia virus. Totals are calculated based upon %total $CD8^+$ $tetramer^+$ cells detected in boosted and non-boosted $CD8^{-/-}$ recipient mice by flow cytometry. Data are representative of at least 2 experiments with 4–6 animals per group. $^*P<0.05$; $^{**}P<0.01$.


A
SYNTGRFPPL targets
KYGRLFNEI targets
Percent Specific Lysis
**
NS
Naïve CD8 T cells
1 week acute effectors
B
CD8 T cell specificity
Naïve
Kd/SYNTGRFPPL
Kd/GFNKLRSTL
1:1 transfer of Kd/SYNTGRFPPL Kd/GFNKLRSTL
SYNTGRFPPL stimulation
GFNKLRSTL stimulation
KYGRLFNEI stimulation
TNFα
IFNγ
0.16 ± 0
1.74 ± 2.5
1.42± 0.1
2.49 ± 0.6
0.053 ± 0.03
2.96± 0.3
5.49± 0.33
8.05 ± 1.5
0.0165 ± 0.02
4.73 ± 1.9
2.26± 1
5.11 ± 0.6
C
SYNTGRFPPL targets/Kd-SYNTGRFPPL effectors
GFNKLRSTL targets/Kd-GFNKLRSTL effectors
KYGRLFNEI targets/Kd-SYNTGRFPPL effectors
KYGRLFNEI targets/Kd-GFNKLRSTL effectors
Percent Specific Lysis
Transfer of naïve CD8 T cells
No boost
Recall
Transfer of effectors into individual recipients
SYNTGRFPPL targets
GFNKLRSTL targets
KYGRLFNEI targets
No boost
Recall
1:1 transfer of effectors into common recipient
D
No Boost
Vaccinia Boost
Totoal number of tetramer-specific CD8 T cells
Kd/GFNKLRSTL CD8+ T cells
Kd/SYNTGRFPPL CD8+ T cells

ability to recall to antigen as memory cells is severely impaired, resulting in secondary $CD8^{-/-}$ recipients developing MAIDS, similar to that of control LP-BM5-infected $CD8^{-/-}$ mice (Fig. 5D).

Discussion

In this study, we have shown that although acute K^d/SYNTGRFPPL-specific CD8 T cells acquired similar effector functional abilities compared to canonical K^d/GFNKLRSTL-specific $C62L^{low}$ effector CD8 T cells, they maintained moderate/high surface levels of CD62L expression (Fig. 2B, Supplementary Fig 2). Despite the higher expression levels of CD62L, these acute effector CTLs were indeed able to protect LP-BM5-susceptible $CD8^{-/-}$ mice during infection with this retrovirus, as we have previously published (Ho and Green, 2006b) (Fig. 5A and Supplementary Fig. 3A–D). However, we found that, although long-lived memory K^d/SYNTGRFPPL-specific CD8 T cells were able to persist without the requirement of antigen (Fig. 2A), they were fundamentally impaired in their ability to mount cytokine responses or lyse antigen-pulsed target cells during antigen-specific recall (Figs. 2B and 4B–C). Transfer of long-lived K^d/SYNTGRFPPL-specific CD8 T cells into $CD8^{-/-}$ recipients infected with LP-BM5 confirmed the apparent lack of function in the memory population, as LP-BM5-infected $CD8^{-/-}$ mice receiving these long-lived K^d/SYNTGRFPPL-specific CD8 T cells developed full-blown MAIDS (Fig. 5B–C and Supplementary Fig. 4A–D).

Typically, stimulation of primed antigen-specific $CD44^{high}$ T cells with cognate antigen in vitro results in the downregulation of CD62L as the cells acquire effector function. Indeed, the majority of $CD62L^{low}$ K^d/GFNKLRSTL-specific acute effector T cells were able to co-produce cytokines IFNγ and TNFα after 9 days of in vitro expansion (Fig. 3B). However, unlike acute K^d/GFNKLRSTL-specific effector T cells, after culture in identical conditions but with SYNTGRFPPL peptide, the majority of IFNγ and TNFα co-producing SYNTGRFPPL-specific T cells expressed moderate/high levels of CD62L (Supplemental Fig. 1C and Fig. 3A). Recent reports have associated the cleavage of CD62L with rapid and efficient viral clearance (Richards et al., 2008), perhaps suggesting a predisposing factor, if not a mechanism, of relevance to our observation that long-lived $CD62L^{moderate/high}$ K^d/SYNTGRFPPL-specific CD8 T cells are poorly able to recall against antigen or protect secondary $CD8^{-/-}$ recipients during LP-BM5 infection. The possibility exists that the IFNγ and TNFα co-producing acute effector $CD62L^{moderate/high}$ population were unable to shed CD62L, in comparison to acute $CD62L^{low}$ effector CD8 T cells generated in identical conditions against a conventional antigen (K^d/GFNKLRSTL-specific CD8 T cells).

However, recently, it has been reported that the generation of protective functional memory T cells occurs irrespective of CD62L expression and is more dependent upon the anatomical location of memory T cells during secondary encounter with antigen (Kedzierska et al., 2007). Although acute K^d/SYNTGRFPPL-specific effector CD8 T cells expressed moderate/high levels of CD62L, they were still capable of cytolysis and effector cytokine production during stimulation with peptide in vitro. The inability of long-lived K^d/SYNTGRFPPL-specific CD8 T cells to protect $CD8^{-/-}$ recipient mice may not be simply due to CD62L expression, as acute and long-lived K^d/SYNTGRFPPL-specific CD8 T cells had similar expression levels of CD62L, compared to naive cells (Supplementary Fig. 2). Although the moderate/high CD62L expression on acute effectors may be predictive of a memory response of poor quality, CD62L expression alone may thus not be directly linked to the impaired nature of the memory CD8 T cell response against SYNTGRFPPL. With regard to the generation of highly functional memory CD8 T cell responses using vaccinia-based expression vectors, the results presented here are interesting to consider in light of the recent publication by Yewdell (2010). It was shown that inflammatory vectors such as vaccinia provide far greater amounts of vector antigen for resident APCs, outcompeting the numbers of CD8 T cells primed against weaker or less abundant antigens (Yewdell, 2010). In terms of our model, the possibility remains that the memory response to SYNTGRFPPL could be rescued by using a less inflammatory vector during recall.

The cardinal feature of memory CD8 T cells is the ability to rapidly recall against antigen in a qualitatively and quantitatively greater capacity than the acute effector CD8 T cell response. Several studies have implicated that the qualitative functions of the CD8 T cell response, not the magnitude, influence the clinical outcome during progression to AIDS (Almeida et al., 2007; Critchfield et al., 2008; Ferre et al., 2009). In relation to targeting CTL responses against cryptic epitopes in HIV, the data obtained suggest that, at least in our system, although a cryptic antigen elicits a protective acute CD8 T cell response, there exists the potential that there may be a decrease in the qualitative effector functions of memory CD8 T cells during recall. In an analogous fashion, perhaps CTL responses against certain highly conserved HIV-encoded cryptic or conventional epitopes may evolve into functionless memory cells. Indeed, we have previously shown that acute SYNTGRFPPL-specific CTL secrete effector cytokines and induce perforin-mediated lysis of target cells (Ho and Green, 2006b; Rutkowski et al., 2009), resulting in protection of susceptible mice. However, long-lived SYNTGRFPPL-specific CD8 T cells were unable to lyse target cells and confer protection during infection with LP-BM5 (Figs. 4C and 5). Our system presents an opportunity to examine in detail possible therapeutic strategies designed at boosting the memory CD8 T cell response against the cryptic epitope SYNTGRFPPL. With respect to therapeutic vaccine strategies, these studies suggest a basis for the rational design and analysis of protective vaccines that elicit polyfunctional and long-lasting protective CD8 T cell responses.

The ability of CD8 T cells to respond effectively against HIV-associated antigens during infection is impaired partially due to the emergence of HIV-1 quasispecies lacking peptide sequences previously recognized by protective CD8 T cells, ultimately resulting in diminished control of the virus set point and progression to AIDS. Reversion to the immunodominant epitopes after transmission to MHC-disparate hosts in humans (Friedrich et al., 2004; Leslie et al., 2004), and replicative deficiencies of escaped mutants during challenge of macaques with SIV compared to wild-type strains (Friedrich et al., 2004) suggest that virus escape from immune pressure exacts a fitness cost to the virus. Nonetheless, virus escape from immune pressure has impeded the success of therapeutic measures targeted at boosting the endogenous CD8 T cell-mediated response to control viral levels. However, recent studies have demonstrated the presence of highly conserved immunodominant cryptic epitopes located within alternate reading frames of the viral genome (Bansal et al., 2010; Cardinaud et al., 2004; Maness et al., 2007). Although immune pressure against cryptic epitopes may also be significant, genetic constraints may exist, resulting in a relatively broad array of highly conserved immunodominant cryptic epitopes (Cardinaud et al., 2004; Ho and Green, 2006a; Mayrand and Green, 1998). Collectively, these studies highlight the previously underappreciated potential of using cryptic epitopes in HIV-1 vaccines and underscore the importance of fully characterizing the recall response against cryptic antigens using the model employed here to directly test protection after priming and boosting with relevant cryptic epitope-based vaccines.

Materials and methods

Mice

Female BALB/c w.t. mice (6–8 weeks old) were purchased from the National Cancer Institute (NCI, Bethesda, MD). Breeding pairs of $CD8^{-/-}$ knockout mice on the BALB/c background were kindly

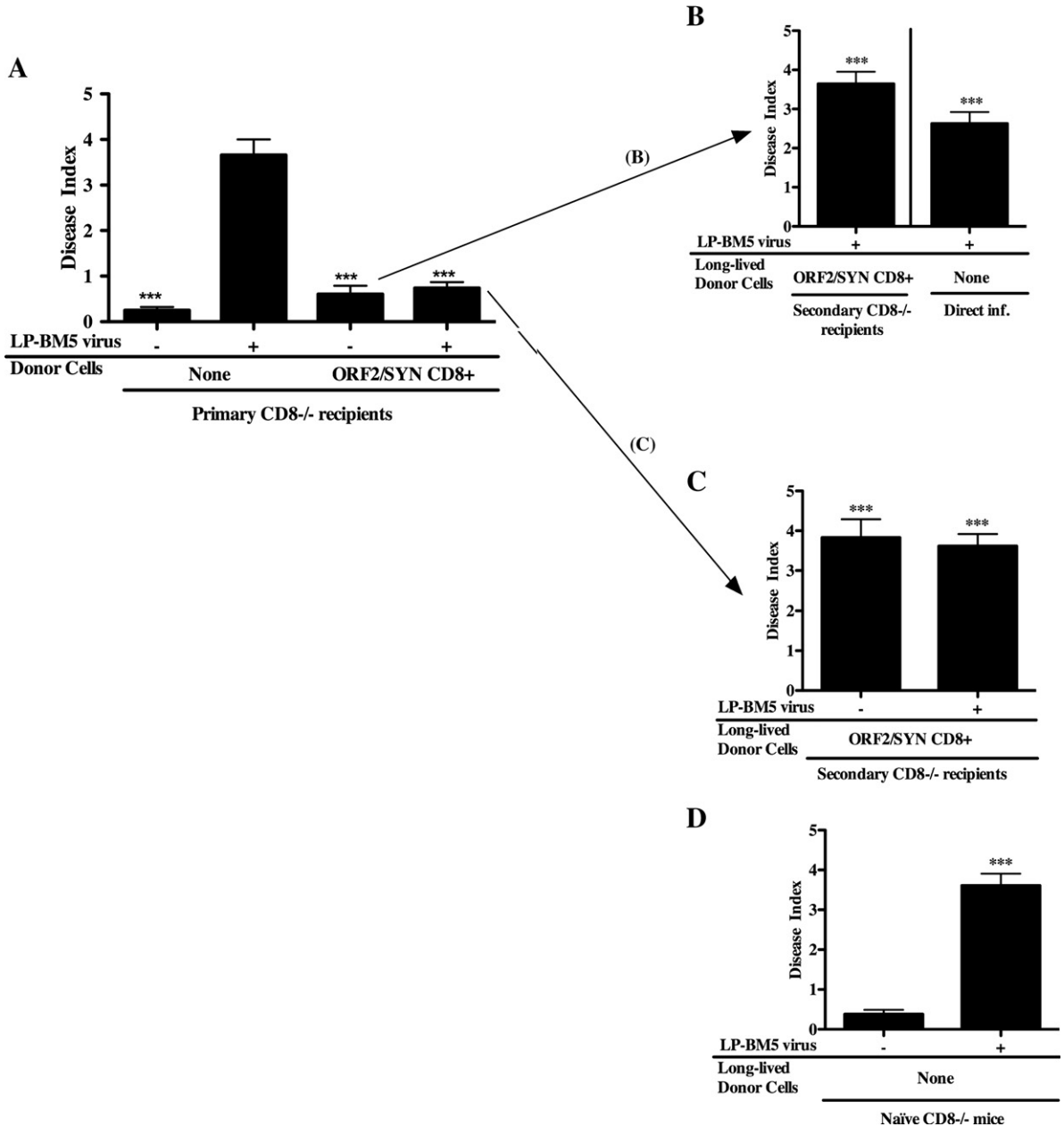


Fig. 5. Unlike acute effector K^d/SYNTGRFPPL-specific CD8 T cells, long-lived K^d/SYNTGRFPPL-specific CD8 T cells are unable to protect susceptible $CD8^{-/-}$ recipient mice during LP-BM5 disease. (A) Extent of disease following infection of primary $CD8^{-/-}$ recipient mice. Primary $CD8^{-/-}$ recipient mice were either infected or non-infected with LP-BM5 retrovirus and administered acute K^d/SYNTGRFPPL-specific effector CD8 T cells (ORF2/SYN $CD8^+$) 11 weeks prior. ***$P<0.001$ compared to $CD8^{-/-}$ mice infected with LP-BM5 only (second bar from the left). (A–B) CD8 T cells were isolated from either non-infected or LP-BM5-infected mice that received acute ORF2/SYN $CD8^+$ 11 weeks prior, as indicated by arrow. (B) Secondary transfer of antigen-independent long-lived ORF2/SYN $CD8^+$ to $CD8^{-/-}$ mice infected with LP-BM5 retrovirus (left histogram). Alternatively, a group of non-infected primary $CD8^{-/-}$ recipients that received ORF2/SYN $CD8^+$ T cells 11 weeks prior were directly infected with LP-BM5 retrovirus (direct inf.) (right histogram). (C) Secondary transfer of antigen-experienced long-lived ORF2/SYN $CD8^+$ to $CD8^{-/-}$ mice infected with LP-BM5 retrovirus. (D) Parallel disease of naive $CD8^{-/-}$ control mice infected at the same time as the infection of the secondary recipients. The disease index of $CD8^{-/-}$ mice either infected or non-infected with LP-BM5 retrovirus that did not receive donor acute effector or long-lived ORF2/SYN $CD8^+$ T cells. ***$P<0.001$ compared to non-infected $CD8^{-/-}$ mice that did not receive donor cells in panel D.

provided by P. Stuart (Washington University, St. Louis, MO) and T. Mak (Ontario Cancer Institute, Toronto, Canada). Mice were bred and housed in specific pathogen-free conditions in the Dartmouth Medical School Animal Resource Center. All experimental procedures were approved by and performed under the requirements set forth by the AAALAC accredited Animal Care and Use Program of Dartmouth College.

Cell lines, viruses, and reagents

The P815B mouse mastocytoma cell line (H-2^d), provided by J. Bennink (NIH/NIAID, Bethesda, MD), was maintained in RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 30 μg/ml penicillin, 20 μg/ml streptomycin, and 33 μg/ml gentamicin. The murine retrovirus LP-BM5, originally provided by J. W. Hartley and H. C. Morse (National Institutes of Health/National Institute of Allergy and Infectious Diseases, Bethesda, MD), was propagated in our laboratory as previously described (Green et al., 2008; Klinken et al., 1988). Vac-DG, the recombinant vaccinia virus with the *gag* gene of the LP-BM5 retrovirus inserted, was generated as previously described (Schwarz and Green, 1994). Vac-M2, the recombinant vaccinia virus with the gammaherpesvirus latent gene inserted, generated by A. McCrae and J. Stewart (University of Liverpool, UK), was a generous gift from E. Usherwood (Dartmouth College). The LP-

BM5 ORF2 peptide (SYNTGRFPPL) was purchased from Invitrogen (Carlsbad, CA) at >95% purity. The H-2K^d-restricted gammaherpesvirus latent peptide M2$_{91-99}$ (GFNKLRSTL) and H-2K^d-restricted vaccinia-specific peptide (KYGRLFNEI) were purchased from New England Peptides (Gardner, MA) at 95% purity. Tetramer, consisting of K^d folded with SYNTGRFPPL peptide and labeled with allophycocyanin, was provided by the NIH Tetramer Core Facility (Atlanta, GA). The allophycocyanin-labeled tetramer, consisting of K^d folded with GFNKLRSTL, was a generous gift from E. Usherwood.

Infection, rechallenge, and adoptive transfer experiments

For all LP-BM5 infections, mice were infected intraperitoneally (i.p.) with the indicated amounts of LP-BM5 retrovirus. For rechallenge experiments, immune or naive mice were injected i.p. with 3×10^5 pfu of Vac-DG 5 days prior to harvesting splenocytes for immunological readouts or in vivo cytotoxicity assays. For experiments using acute effector K^d/SYNTGRFPPL-specific CD8 T cells, antigen-specific T cells were enriched by depletion of CD19$^+$ cells using magnetic beads (Miltenyi, Auburn CA) from BALB/c mice primed 1 week prior with 3×10^7 pfu Vac-DG and approximately $1–2\times10^7$ splenocytes/recipient were transferred into CD8$^{-/-}$ recipient mice. For the generation of long-lived K^d/SYNTGRFPPL-specific or K^d/GFNKLRSTL-specific CD8 T cells, w.t. BALB/c mice were immunized with 3×10^7 pfu of Vac-DG or Vac-M2 as previously reported (Ho and Green, 2006b). Approximately 3 weeks post-priming, antigen-sensitized splenocytes were isolated from Vac-DG or Vac-M2-primed mice followed by incubation with the 10 ng/ml of SYNTGRFPPL or GFNKLRSTL peptide, respectively, for 6 days and an additional 3 days of culture with 5U/ml of rIL-2 (Cetus Corporation, Everyville, CA). Prior to adoptive transfer into BALB/c CD8$^{-/-}$ recipients, CTLs were characterized for antigen specificity by staining with tetramer. SYNTGRFPPL-specific cytotoxicity and effector cytokine production of IFNγ, TNFα, and IL-2 were assessed by using the standard ^{51}Cr release assay (Schwarz and Green, 1994) and intracellular cytokine staining, respectively. For four consecutive 3-day intervals, CTLs containing approximately 5×10^5 ORF2/SYNTGRFPPL-specific CTL (enumerated by tetramer staining) were transferred intravenously into naive or CD8$^{-/-}$ recipients infected with LP-BM5 3 days prior.

Chromium release cytotoxicity assays

In vitro cytolytic activity was determined using a standard ^{51}Cr release assay with P815B target cells pulsed with 200 μCi of ^{51}Cr, followed by pulsing with or without 100 ng/ml of either SYNTGRFPPL, GFNKLRSTL, or KYGRLFNEI synthetic peptides at 37 °C for 30 min in RPMI supplemented with 10% calf serum, 30 μg/ml penicillin, and 20 μg/ml streptomycin. Target cells were ad-mixed at the indicated ratios with effector CD8 T cell populations and incubated at 37 °C for 6 h. Percent specific lysis was calculated as previously described (Schwarz and Green, 1994).

Intracellular cytokine staining

Intracellular staining for cytokines IFNγ, TNFα, and IL-2 was performed as previously described (Rutkowski et al., 2009). Cells were cytoplasmically stained with APC-conjugated-anti-mouse IFNγ, PE-conjugated anti-mouse TNFα, or FITC-conjugated anti-mouse IL-2, or the appropriate isotype control mAbs. All antibodies were from either BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA).

IFNγ enzyme-linked immunospot assay (ELISPOT)

Lymphocytes secreting IFNγ were quantified using a standard ELISPOT assay. Flat-bottomed 96-well HTS nitrocellulose plates (Millipore, Bedford, MA) were coated with rat anti-mouse IFNγ capture antibody (BD Pharmingen) overnight at 4 °C. Plates were washed and blocked with media containing 10% FBS for 2 h at 37 °C. Splenocytes from immune and non-immune mice were plated at 5×10^5 cells/well in triplicate together with 10^6 irradiated (3000 rad) syngeneic splenocytes and 10 μg/ml stimulating SYNTGRFPPL, or irrelevant GFNKLRSTL, peptide and 10 U/ml rIL-2 for 48 h at 37 °C. After 48 h, plates were washed with PBS containing 0.5% Tween-20 (0.5% PBS) followed by incubation with 2 μg/ml biotinylated rat anti-mouse IFNγ detecting mAb at 4 °C for a minimum of 2 h. Plates were washed and spots were visualized by the addition of streptavidin–alkaline phosphatase for 1 h at room temperature followed by washing and addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tratazolium (BCIP/NBT substrate; Sigma) for 30–60 min at room temperature.

Tetramer staining

ORF2/SYNTGRFPPL-specific CTLs were incubated with anti-FcγII/III on ice for 10 min. Cells were then incubated with APC-conjugated K^d/SYNTGRFPPL tetramer or control APC-conjugated K^d/GFNKLRSTL at room temperature for 1 h, followed by labeling with PercP-anti-CD8α, and either PE-anti-CD127, or PE-anti-CD44, or PE-anti-CD122; and FITC-anti-CD43 (1B11 clone) or FITC-anti-CD62L for 20 min on ice. Stained cells were then washed thoroughly and analyzed on a FACSCalibur as previously stated.

In vivo cytotoxicity assay

In vivo measurement of antigen-specific CD8 cytolytic activity was performed as previously described (Barber et al., 2003; Fuse and Usherwood, 2007). Briefly, syngeneic BALB/c splenocytes were pulsed with 2 μg/ml of the indicated peptides, or no peptide, for 1 h at 37 °C in serum-free media. Cells were washed and labeled with the following cellular dyes: 2 μM or 0.2 μM carboxyfluorscein succinimidyl ester (CFSE; Invitrogen, Eugene, OR) in serum free media for 10 min at room temperature, or 10 μM Cell Tracker Orange, chloromethyl-benzoyl-aminotetramethyl-rhodamine (CMTMR; Invitrogen) for 30 min in media with 10% serum at 37 °C, followed by an additional 30-min incubation at 37 °C in media with 10% serum without the cellular dye. After the incubation periods, cells were washed extensively, counted, and combined at a 1:1:1 ratio, with an approximate total of $0.8–2\times10^7$ cells injected intravenously into recipient mice. Sixteen hours post-transfer, mice were sacrificed and spleen cell suspensions were collected, counted, and incubated with 25 μg/ml of 7-amino-actinomycin D (7-AAD; BD Pharmingen) at room temperature for 10 min to exclude all non-viable splenocytes. Specific lysis was calculated using the following formulas: ratio = (number of peptide labeled targets/number of non-labeled targets); percentage of specific lysis = $(1-(\text{ratio immunized/ratio naive})\times100)$.

LP-BM5 disease measurement

For analysis of LP-BM5-induced activational disease parameters, lymphoproliferation was determined by measuring spleen weight, and hypergammaglobulinemia was assessed via measuring serum levels of IgG2A and IgM in a standard ELISA assay, as previously described in detail (Green et al., 2008; Li and Green, 2006), from serum previously separated from peripheral blood of sacrificed mice. Immunodeficiency was measured using ConA and LPS mitogen-induced proliferation assays of isolated splenocytes as previously described (Green et al., 2008; Li and Green, 2006). For calculation of viral load, mRNA encoding the BM5 defective (BM5def) and ecotropic (BM5eco) *gag* genes was amplified from purified splenic RNA by separate real-time RT-PCR assays as previously described (Cook et al., 2003).

Calculation of disease index

Disease index was calculated as previously described (Rutkowski et al., 2009). Briefly, percent disease of all experimental mice for each disease parameter (splenomegaly, LPS and ConA mitogen response, and serum levels of IgG2A and IgM) was calculated using the formula: ((experimental value − mean value of non-infected controls)/(mean value of LP-BM5-infected controls − mean value of non-infected controls))×100. Mice with 0% or calculated "negative" disease for any one parameter were assigned a value of 0, mice with 1–20% disease were assigned a value of 0.5, mice with 21–40% disease were assigned a value of 1, mice with 41–60% disease were assigned a value of 2, mice with 61–80% disease were assigned a value of 3, mice with 81–100% disease were assigned a value of 4, and mice with greater than 100% disease were assigned a value of 5. Values for all parameters were combined for each experimental set of mice and graphed.

Statistics

Prism (GraphPad; San Diego, CA) was used for all statistical tests of significance (P values of ≤ 0.05).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.11.013.

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