

# CD4<sup>+</sup> T cell regulation of CD25 expression controls development of short-lived effector CD8<sup>+</sup> T cells in primary and secondary responses

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Both CD4<sup>+</sup> T cell help and IL-2 have been postulated to “program” activated CD8<sup>+</sup> T cells for memory cell development. However, the linkage between these two signals has not been well elucidated. Here we have studied effector and memory CD8<sup>+</sup> T cell differentiation following infection with three pathogens (*Listeria monocytogenes*, vesicular stomatitis virus, and vaccinia virus) in the absence of both CD4<sup>+</sup> T cells and IL-2 signaling. We found that expression of CD25 on antigen-specific CD8<sup>+</sup> T cells peaked 3–4 days after initial priming and was dependent on CD4<sup>+</sup> T cell help, likely through a CD28:CD80/86 mediated pathway. CD4<sup>+</sup> T cell or CD25-deficiency led to normal early effector CD8<sup>+</sup> T cell differentiation, but a subsequent lack of accumulation of CD8<sup>+</sup> T cells resulting in overall decreased memory cell generation. Interestingly, in both primary and recall responses KLRG1<sup>high</sup> CD127<sup>low</sup> short-lived effector cells were drastically diminished in the absence of IL-2 signaling, although memory precursors remained intact. In contrast to previous reports, upon secondary antigen encounter CD25-deficient CD8<sup>+</sup> T cells were capable of undergoing robust expansion, but short-lived effector development was again impaired. Thus, these results demonstrated that CD4<sup>+</sup> T cell help and IL-2 signaling were linked via CD25 up-regulation, which controls the expansion and differentiation of antigen-specific effector CD8<sup>+</sup> T cells, rather than “programming” memory cell traits.

infection | memory

Although recent findings have advanced our knowledge of the factors necessary to generate optimal memory CD8<sup>+</sup> T cell responses, a full understanding of the signals required remains elusive. CD8<sup>+</sup> T cell responses to acute viral or bacterial infections are characterized by three phases. Upon recognition of a specific antigenic epitope, T cells undergo massive proliferation and acquisition of effector functions. Pathogen-specific CD8<sup>+</sup> T cells can expand close to 10<sup>5</sup>-fold from a population as small as a few hundred precursors (1). These effector T cells have altered chemokine and homing receptor expression enabling their migration through all peripheral tissues (2, 3). On further antigenic stimulation, the effector CD8<sup>+</sup> T cells will kill infected target cells through several mechanisms thereby limiting pathogen growth and dissemination. After this stage, effector CD8<sup>+</sup> T cells enter a contraction phase where a majority of the effector cells undergo apoptosis leaving only 5–20% of the cells present at the peak of expansion (4). The last stage is characterized by the maintenance of a heterogeneous population of long-lived, stable CD8<sup>+</sup> memory T cells (5). These remaining cells are characterized as stem-cell like because they retain telomerase expression, the ability to self-renew, a high proliferative capacity, and multipotency (5). In acute infections, the persistence of these memory T cells relies on the presence of the common gamma-chain ( $\gamma_c$ ) cytokines IL-7 and IL-15 (6, 7). Upon secondary exposure to the specific antigen, memory CD8<sup>+</sup> T cells undergo a more rapid expansion and production of effector cy-

tokines compared to the primary response, which results in rapid control of infection.

Unfortunately, these cardinal rules of memory CD8<sup>+</sup> T cell function do not account for the extensive functional and phenotypic complexity of subsets that is known to exist (5). Additionally, exactly when each subset differentiates from the original naïve precursor remains controversial (8). Recent work has demonstrated that a single naïve antigen-specific CD8<sup>+</sup> T cell can give rise to all of the effector and memory subsets observed (9). One theory suggests that heterogeneity occurs as early as the first asymmetric division of a naïve cell, where one daughter cell is “fated” to become effector-like through the up-regulation of effector molecules and expression of differentiation markers. Meanwhile, the sister cell retains the ability to differentiate into a memory-like cell (10). It is unclear whether these lineages are set in stone or whether manipulation of cell fate can occur through the presence or lack of secondary stimulation. Recent evidence indicates the latter scenario is likely true because cells that have up-regulated granzyme B (and therefore would be considered effector-like) maintained the ability to form long-lived memory cells (11).

Population heterogeneity continues throughout the expansion phase, as the ability to form memory cells can be correlated with the expression patterns of CD127 and the killer cell lectin-like receptor G1 (KLRG1) (12–14). Briefly, KLRG1<sup>high</sup> CD127<sup>low</sup> CD8<sup>+</sup> T cells are considered to be more terminally differentiated and die after clearance of the infection and therefore are referred to as short-lived effector cells (SLEC). KLRG1<sup>low</sup> CD127<sup>high</sup> CD8<sup>+</sup> T cells are referred to as memory precursor effector cells (MPEC) because they possess effector properties, but retain the ability to differentiate into a long-lived memory population. The factors regulating the formation of the SLEC and MPEC populations remain ill-defined. It is well established that three signals are necessary for the activation of naïve CD8<sup>+</sup> T cells: (i) TCR stimulation, (ii) costimulation, and (iii) inflammatory cytokines (15), but how variations in these signals may orchestrate effector and memory heterogeneity is not clear. Interestingly, alterations in the amount of antigenic exposure do not seem to skew effector cell differentiation, but the inflammatory milieu is able to alter the SLEC/MPEC ratio (12, 14, 16).

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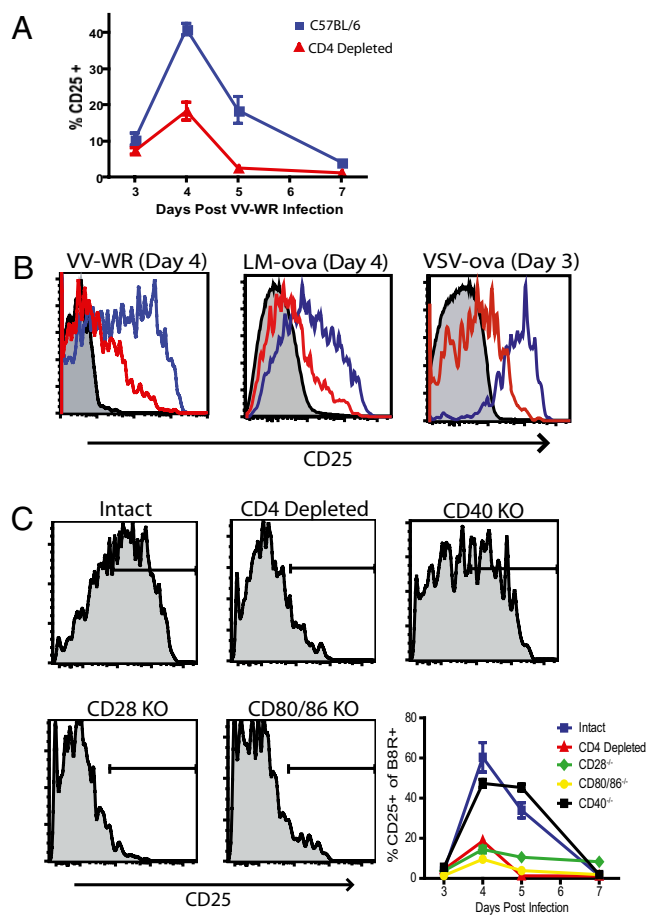
Based upon this, we explored whether other known regulators of effector and memory CD8<sup>+</sup> T cell differentiation, namely CD4<sup>+</sup> T cell help and IL-2 signaling, are critical for the development of SLEC and MPEC populations.

Costimulatory signals driven by CD4<sup>+</sup> T cells have been shown to be crucial for the optimal differentiation of memory CD8<sup>+</sup> T cells in various models (17–19). However, controversy remains as to precisely when CD4<sup>+</sup> T cell help is required for memory CD8<sup>+</sup> T cell differentiation. Depending on a number of variables in different infection models, CD4<sup>+</sup> T cells are required during priming, maintenance, or recall and, in some cases, CD4<sup>+</sup> T cell help can be nonessential (17–21). An important variable that alters the requirement for CD4<sup>+</sup> T cell help is the pathogen that is under study. For example, the CD8<sup>+</sup> T cell response to vesicular stomatitis virus (VSV) infection is largely independent of CD4<sup>+</sup> T cell help. In the case of the CD8<sup>+</sup> T cell response to *Listeria monocytogenes* (LM) infection, some reports indicate a CD4<sup>+</sup> T cell requirement for primary expansion (21, 22) although others do not (18, 19). However, even when CD4<sup>+</sup> T cell help is apparently not required for primary expansion, the “helpless” memory CD8<sup>+</sup> T cells respond poorly to challenge (17–19), but there is disagreement in the literature on this point (21, 22). Additionally, in the absence of CD4<sup>+</sup> T cell help, immune responses to intranasal infection with vaccinia virus-Western Reserve (VV-WR) or murine  $\gamma$ -herpesvirus generate normal primary responses, but secondary recall is inhibited through the up-regulation of PD-1 (23). Although the critical signal provided by CD4<sup>+</sup> T cell help appears to be the ligation of CD40 on antigen presenting cells, IL-2 production by CD4<sup>+</sup> T cells may also play a role (24–26). Additionally, previous studies indicate that CD4<sup>+</sup> T cell help and IL-2 signaling during priming, although not shown to be synonymous, are not required for the primary CD8<sup>+</sup> T cell response to infection but are essential for the formation of a functional memory population (27, 28). Nonetheless, the mechanism by which CD4<sup>+</sup> T cells impinge on CD8<sup>+</sup> effector and memory T cell subset development remains unclear.

The results presented here demonstrated that optimal differentiation of the SLEC population during primary and secondary CD8<sup>+</sup> T cell responses required the expression of CD25 and that CD4<sup>+</sup> T cell help, possibly through CD28 costimulation, regulated the expression of CD25 on the pathogen-specific CD8<sup>+</sup> T cells. Thus, the helpless condition or the absence of CD25 resulted in a marked reduction of the primary CD8<sup>+</sup> T cell response that was largely restricted to the SLEC subset. However, the resulting memory CD8<sup>+</sup> T cells readily responded to secondary challenge. Our findings supported a role for CD4<sup>+</sup> T cells in promoting IL-2 driven effector CD8<sup>+</sup> T cell expansion and survival, but do not support the notion that these factors affect the functionality of memory CD8<sup>+</sup> T cells.

## Results

**CD4<sup>+</sup> T Cell Help Is Critical for the Up-Regulation of CD25 by Antigen-Specific CD8<sup>+</sup> T Cells.** We wished to test whether a link exists between CD4<sup>+</sup> T cell help and CD25 expression by CD8<sup>+</sup> T cells. To account for potential variability between different systems, we examined responses to LM or VSV expressing ovalbumin (LM-ova; VSV-ova), and VV-WR. C57BL/6 CD8<sup>+</sup> T cells up-regulated CD25 transiently with peak expression at approximately 4 days after infection regardless of the pathogen studied (Fig. 1A and B). In contrast, pathogen-specific CD8<sup>+</sup> T cells in mice treated with anti-CD4 mAb or in MHC class II<sup>-/-</sup> mice only weakly up-regulated CD25 (Fig. 1A and B and Fig. S1). As past studies have shown that CD40 deficient mice have similar responses to helpless mice and agonistic anti-CD40 antibodies can substitute for CD4<sup>+</sup> T cell help, we tested whether CD40<sup>-/-</sup> mice lacked the ability to up-regulate CD25 (25, 29). Surprisingly, VV-specific CD8<sup>+</sup> T cells from CD40-deficient animals expressed high levels of CD25, indistinguishable from those in normal mice (Fig. 1C). In contrast, only minimal up-regulation of CD25 was observed in CD8<sup>+</sup> T cells from both CD28-deficient and CD80/86-deficient mice (Fig. 1C). The decreased



**Fig. 1.** CD4<sup>+</sup> T cell help is required for the expression of CD25 by antigen-specific CD8<sup>+</sup> T cells. (A) Time-course showing the percentage of B8R/K<sup>b</sup>-specific CD8<sup>+</sup> T cells expressing CD25 for mice infected with VV-WR in the presence (blue) or absence (red) of CD4<sup>+</sup> T cells for 7 days p.i. (B) Representative histograms show the peak expression of CD25 following infection with VV-WR, LM-ova, or VSV-ova in the presence (blue line) or absence (red line) of CD4<sup>+</sup> T cells. Shaded histograms = isotype control. (C) Representative histograms and time-course showing CD25 expression on B8R/K<sup>b</sup>-specific CD8<sup>+</sup> T cells from intact (blue) or CD4-depleted WT mice (red), CD40<sup>-/-</sup> mice (black), CD28<sup>-/-</sup> mice (green), or CD80/86<sup>-/-</sup> mice (orange) four days p.i.

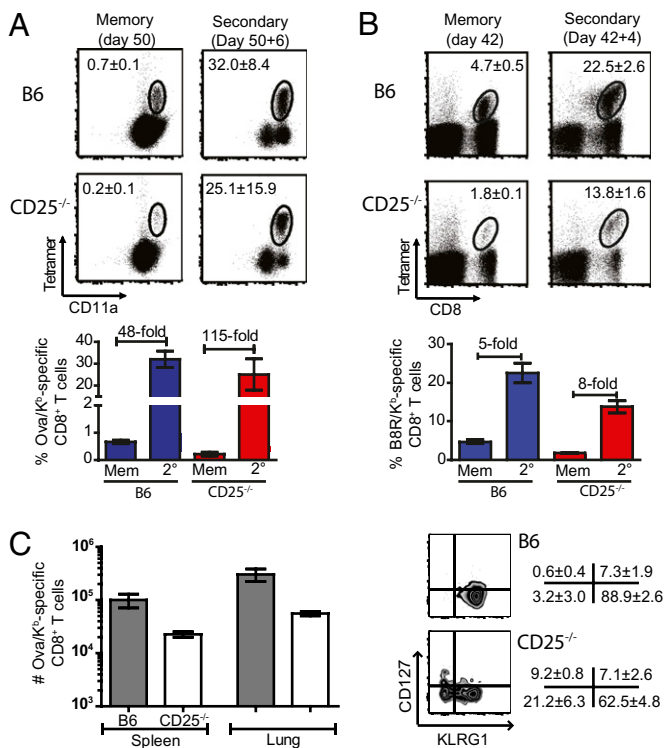
CD25 expression could be a direct result of CD28:CD80/86 signals but might also be a consequence of decreased IL-2 production because CD28:CD80/86 signals are known to regulate IL-2 expression which in turn can regulate CD25 expression (30). To test this hypothesis, helpless mice were treated with 15,000U of rhIL-2 twice a day and subsequently CD25 expression on day 4 after VV infection was determined. Interestingly, rhIL-2 treatment was able to restore CD25 expression by VV-specific CD8<sup>+</sup> T cells (Fig. S2). Thus, CD4<sup>+</sup> T cell help plays an important role in regulating the expression of CD25 on pathogen-specific CD8<sup>+</sup> T cells during infection potentially through CD28-CD80/86 mediated expression of IL-2 from CD4<sup>+</sup> T cells.

### CD25 Expression Is Necessary for Optimal Expansion of CD8<sup>+</sup> T Cells.

As helpless CD8<sup>+</sup> T cells were unable to up-regulate CD25, we examined whether primary responses were compromised in anti-CD4 mAb treated mice. Following infection, antigen-specific CD8<sup>+</sup> T cells from all groups of mice underwent an initial robust expansion, but responding CD8<sup>+</sup> T cells from LM-ova and VV-WR infected anti-CD4 mAb treated hosts were unable to sustain expansion (Fig. 2A and B). In contrast, CD4-depleted VSV-ova infected mice mounted a primary response equivalent to that of







**Fig. 6.** CD25-deficient memory CD8<sup>+</sup> T cells are able to mount a robust secondary response. CD25<sup>-/-</sup>/WT mixed chimera mice were primed with 10<sup>3</sup> CFU of LM-ova (A) or 2 × 10<sup>6</sup> PFU of VV-WR (B) and left for more than 42 days and challenged with 5 × 10<sup>4</sup> CFU of LM-ova or 10<sup>6</sup> CFU of LM-B8R, respectively. Mice were subsequently bled to monitor the magnitude of the pathogen-specific CD8<sup>+</sup> T cell response. Representative dot plots show the prechallenge frequency of antigen-specific CD8<sup>+</sup> T cells and the peak recall response. The bar graph shows the average expansion of the pathogen-specific CD8<sup>+</sup> T cells at the indicated times and relative fold expansion of each population. (C) WT (CD45.1<sup>+</sup>) and CD25<sup>-/-</sup> (CD45.2<sup>+</sup>) CD8<sup>+</sup> T cells were purified by flow cytometry and then 1,000 Ova/K<sup>b</sup>-specific CD8<sup>+</sup> T cells mixed together and injected i.v. into naïve CD45.1<sup>+</sup>/CD45.2<sup>+</sup> recipient mice. One day later mice were challenged with 5 × 10<sup>3</sup> PFU of Lm-ova and the number of Ova/K<sup>b</sup>-specific CD8<sup>+</sup> T cells was enumerated in the spleen and lungs on day 8 postchallenge. Zebra plots are gated on the Ova/K<sup>b</sup>-specific CD8<sup>+</sup> T cells of WT or CD25<sup>-/-</sup> origin and secondary effector cell subpopulations were determined. Values represent the mean ± 1 SD from four mice and data are representative of two independent experiments.

CD40 independent but required CD4<sup>+</sup> T cells, IL-2, and CD28-mediated costimulation (Fig. 1). CD4<sup>+</sup> T cells and IL-2 were also critical for driving normal primary CD8<sup>+</sup> T cell responses to LM and VV-WR infections (Figs. 2 and 3). Interestingly, CD4<sup>+</sup> T cell help and IL-2 were needed relatively late in the primary response to promote proliferation and perhaps survival because early CD8<sup>+</sup> T cell expansion occurred normally in the absence of CD4<sup>+</sup> T cells or CD25 but was followed by a more dramatic contraction phase (Fig. 3). Thus, it is important to consider the overall kinetics of a response when analyzing potentially controlling factors.

Our findings indicated that one mechanism by which CD4<sup>+</sup> T cells may provide help was through the control of the up-regulation of CD25 expression by recently activated CD8<sup>+</sup> T cells (Fig. 1). The decreased CD25 expression could be a direct result of CD28:CD80/86 signals but might also be a consequence of decreased IL-2 production in the absence of CD4<sup>+</sup> T cells because IL-2 is known to regulate CD25 expression (34). Both are likely the case as engagement of the CD28:CD80/86 pathway causes IL-2 expression (30) and the provision rIL-2 in the absence of CD4<sup>+</sup> T cell help resulted in up-regulation of CD25 expression (Fig. S2 and ref. 34). Either way, in the absence of CD4<sup>+</sup> T cells, CD25 was poorly up-regulated on re-

sponding CD8<sup>+</sup> T cells after VV, VSV, and LM infections. This is in line with previous studies that have demonstrated that CD4<sup>+</sup> T cell help regulates CD25 expression on CD8<sup>+</sup> T cells after HSV-1 infection (35) and DC vaccination (36) and correlated with the CD4<sup>+</sup> T cell dependence of these responses. In contrast, previous studies reported no CD4<sup>+</sup> T cell or CD25 involvement in the primary CD8<sup>+</sup> T cell response to LCMV and LM infections (18, 19, 27, 28), although other studies support a role for CD4<sup>+</sup> T cells in primary CD8<sup>+</sup> T cell responses to these and other immunogens (21, 22). Interestingly, the route of infection may also determine the necessity of CD4<sup>+</sup> T cell help because primary CD8<sup>+</sup> T cell responses were “help” independent when mice were infected i.n. with VV-WR (23), but our current study demonstrated the primary response to i.p. infection with VV-WR was dependent on CD4<sup>+</sup> T cell help (Fig. 2). Interestingly, our data indicated that the early CD8<sup>+</sup> T cell response was independent of CD4<sup>+</sup> T cell help and IL-2, whereas the continued differentiation and expansion typically required both (Figs. 3 and 5). Whether the necessary IL-2 for sustained CD8<sup>+</sup> T cell expansion is derived solely from CD4<sup>+</sup> T cells is not known. Our previous studies showed that autocrine CD8<sup>+</sup> T cell produced IL-2 is not required for maximal expansion but, in fact, appears to dampen the overall magnitude of the response (32). Furthermore, work by Livingstone and colleagues demonstrated that CD4<sup>+</sup> T cells provided the major source of IL-2 for responding antigen-specific CD8<sup>+</sup> T cells (26). In either case, CD25 expression would be required to mediate IL-2 effects and therefore CD4<sup>+</sup> T cells would be involved in the regulation of the CD8<sup>+</sup> T cell response at multiple levels.

Recent work illustrates that substantial heterogeneity exists within the effector CD8<sup>+</sup> T cell population. Originally, memory-precursors were identified by their retention of CD127 expression (37, 38), which has now been refined to use KLRG1 and CD127 expression to identify MPEC and SLEC (12–14), and, in this report, EEC. Only recently have some of the factors regulating the differentiation of the MPEC and SLEC populations been identified. Although earlier work demonstrated that TCR or cytokine signaling alone were insufficient to up-regulate KLRG1 on T cells (39), recent work has shown that IL-12 signaling in the antigen-specific CD8<sup>+</sup> T cells is important for SLEC differentiation and this process is T-bet mediated (12). Furthermore, inflammatory and IL-12 signaling events are temporally linked with TCR engagement (16). The transcription factor Blimp1 has been found to be important in the generation of SLEC (40, 41). Interestingly, Blimp1 expression is regulated by IL-2 in vitro (42) and our data demonstrated that IL-2 mediated signals played an important role in regulating the formation of the SLEC population (Fig. 4). Thus, IL-2 likely mediates early events in the initial differentiation of the SLEC, although IL-2, perhaps through enhancement of Blimp1 expression, acts at later times to further promote the differentiation of more SLECs, perhaps from EEC that express the highest levels of CD25.

Our studies further demonstrated that CD25-deficient pathogen-specific CD8<sup>+</sup> T cells generated an MPEC population during the effector stage, and these cells went on to form a stable, long-lived memory pool (Figs. 3 and 4). Moreover, in the absence of IL-2 driven signals, memory CD8<sup>+</sup> T cells from LM and VV infected mice responded vigorously to a secondary challenge (Fig. 6). However, SLEC development was again dampened just as we observed in the primary response implicating IL-2 in modulation of the primary and secondary responses through enhancing the late differentiation of the SLEC population. Nevertheless, we found little evidence for a helpless phenotype of memory CD8<sup>+</sup> T cells that developed in the absence of CD4<sup>+</sup> T cells or CD25. Previous studies performed with LCMV-specific P14 TCR transgenic CD8<sup>+</sup> T cells lacking CD25 indicate that although cells are capable of proliferating during a secondary response, their overall accumulation is impaired. The authors attributed these findings to a potential increase in cell death (27). In contrast, we observed robust expansion of CD25<sup>-/-</sup> CD8<sup>+</sup> T

cells during the secondary response at multiple time points. It is possible that LCMV secondary responses are dependent upon IL-2 signals, although the infectious models we studied are not because the LCMV results were confirmed by a separate group (28). However, Williams et al. (27) also observed a defect in recall responses to LM-ova in experiments that were very similar to our own. Although we cannot explain these discrepancies, the reproducibility of our results in three separate infection models suggested that imprinting of proliferative capacity during the primary CD8<sup>+</sup> T cell response for the derived memory cells is not a generalizable phenomenon. Therefore, we conclude that CD4<sup>+</sup> T cell help drives CD25 up-regulation and this in turn allows IL-2 to enhance SLEC differentiation during both primary and secondary CD8<sup>+</sup> T cell responses, rather than acting as a “programmer” of the downstream memory response.

## Materials and Methods

**Mice.** All mice were used between 5 and 8 weeks of age for these studies. To generate mixed bone marrow chimeras, B6-Ly5.2 recipient mice were lethally irradiated with approximately 1,000 rads and subsequently injected i.v. with a total of 10<sup>6</sup> bone marrow cells containing a mixture of CD25<sup>-/-</sup> and B6-Ly5.2 at a 2:1 ratio. Mice were left for at least 50 days before infection. All animal protocols were approved by the Animal Care Committee at University of Connecticut Health Center, Farmington, CT, and the Animal Care and Use Program at Dartmouth College, Hanover, NH.

**Experimental Treatment of Mice.** Mice were infected with 10<sup>5</sup> PFU VSV-ova (i.v.), 10<sup>3</sup> CFU LM-ova (i.v), or i.p. infection with 2 × 10<sup>6</sup> VV-WR. For second-

dary infections VV-WR memory mice were infected i.v. with 1 × 10<sup>6</sup> CFU of attenuated (ActA<sup>-</sup> LLO) LM expressing the B8R epitope (LM-B8R), whereas both VSV-ova and LM-ova memory mice were challenged with 5 × 10<sup>4</sup> CFU of LM-ova. CD4<sup>+</sup> T cells were depleted as previously described (21, 23). To supply exogenous IL-2, mice were treated with 15,000 U of recombinant human IL-2 i.p., as previously performed (34).

**Tissue Sample Preparation and Flow Cytometric Analysis.** Mice were either bled at the indicated time points or single cell suspensions of the indicated tissues were prepared by collagenase digestion as previously described (2), and cells were stained as previously described (1, 23). BrdU incorporation was analyzed as previously described (1).

**Adoptive Transfer and Recall of Memory CD8<sup>+</sup> T Cells.** WT (CD45.1) and CD25-deficient (CD45.2) memory CD8<sup>+</sup> T cells were sorted on a FACSAria to >98% purity. 1000 Ova/K<sup>b</sup>-specific memory CD8<sup>+</sup> T cells from each cell population were mixed together and injected i.v. into naïve CD45.1/CD45.2 recipient mice. One day later mice were challenged with 5 × 10<sup>3</sup> PFU of LM-ova and the number of tetramer<sup>+</sup> cells was enumerated in the spleen and lungs on day 8 postchallenge.

**Statistical Analysis.** Statistical significance was determined by a Student's *t* test using Prism 5. Significance was set as any *P* value less than 0.05.

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