

# Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells *in vivo*

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**The naïve and memory T lymphocyte pools are maintained through poorly understood homeostatic mechanisms that may include signaling *via* cytokine receptors. We show that interleukin-7 (IL-7) plays multiple roles in regulating homeostasis of CD8<sup>+</sup> T cells. We found that IL-7 was required for homeostatic expansion of naïve CD8<sup>+</sup> and CD4<sup>+</sup> T cells in lymphopenic hosts and for CD8<sup>+</sup> T cell survival in normal hosts. In contrast, IL-7 was not necessary for growth of CD8<sup>+</sup> T cells in response to a virus infection but was critical for generating T cell memory. Up-regulation of Bcl-2 in the absence of IL-7 signaling was impaired after activation *in vivo*. Homeostatic proliferation of memory cells was also partially dependent on IL-7. These results point to IL-7 as a pivotal cytokine in T cell homeostasis.**

To maintain immunocompetence it is important to sustain the numbers and proportions of T and B lymphocytes in both the naïve and memory compartments. T and B cell populations are independently regulated<sup>1</sup> and the size of the naïve and memory T cell pools appear to be independently maintained<sup>2</sup>. Under normal circumstances, T cell homeostasis is probably mediated by maintaining cell survival and/or proliferation, rather than by regulation of T cell production by the thymus<sup>3</sup>. Naïve T cells require interaction with autologous major histocompatibility complex (MHC) molecules to survive and for low-level proliferation<sup>4–10</sup>. Upon transfer to a lymphopenic host, naïve T cells will increase their proliferation in an effort to reconstitute the host, and this may be one mechanism by which T cell homeostasis is mediated<sup>11,12</sup>. In this model, the proliferation of naïve CD8<sup>+</sup> T cells requires the presence of MHC class I molecules and low-affinity peptides<sup>13–15</sup>. It is believed that the interactions that drive naïve T cell proliferation are similar to those that drive positive selection in the thymus. Although interactions with MHC class I molecules and peptide appear to be important for naïve T cell maintenance, the mechanism by which this interaction regulates survival is not known.

Like naïve T cells, memory T cells increase their rate of proliferation upon transfer to a lymphopenic host<sup>4,16</sup>. However, this homeostatic proliferation is not dependent on MHC molecules<sup>16,17</sup>. Also, memory T cells survive long-term *in vivo* in part *via* continual low-level cell division<sup>9,10,16,17</sup>. The roles of interleukin 2 (IL-2), IL-7 and IL-15 in proliferation of memory T cells after transfer into normal hosts have been examined<sup>18</sup>. CD44<sup>high</sup>-sorted memory phenotype cells proliferate less when the actions of IL-2 and IL-15 are blocked, whereas blocking of IL-2 alone enhances proliferation of memory T cells<sup>18</sup>. These results suggest that IL-15 is involved in inducing the proliferation of memory CD8<sup>+</sup> T cells, whereas IL-2 may play the opposite role by inhibiting proliferation. IL-15 has previously been implicated<sup>18–21</sup> in the proliferation and maintenance of memory CD8<sup>+</sup> T cells. However, inhibition of

IL-7 does not always inhibit proliferation of memory T cells and thus the role of IL-7 in memory T cell proliferation remains unclear.

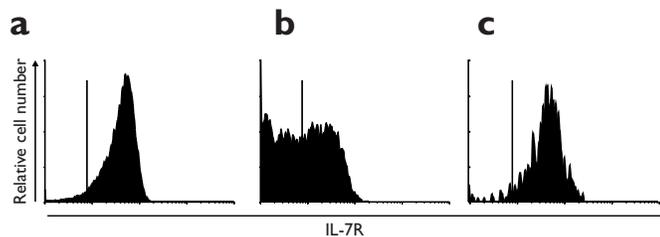
These studies demonstrate that IL-2, IL-15 and possibly IL-7 regulate the proliferation of memory CD8<sup>+</sup> T cells in normal hosts. Whether the same cytokines regulate survival or proliferation of naïve or activated T cells is not known. As with MHC requirements, the cytokine requirements for proliferation of naïve *versus* memory T cells may also be distinct. In this respect IL-7 and IL-4 in combination, both of which bind  $\gamma_c$  receptors, are involved in naïve, but not memory, CD4<sup>+</sup> T cell survival<sup>19</sup>. In the absence of all  $\gamma_c$  receptors, survival of naïve T cell receptor (TCR) transgenic CD4<sup>+</sup> thymocytes is impaired but antigen-driven expansion and memory cell maintenance is not affected<sup>20</sup>. Thus different subclasses, as well as distinct activation stages, of T cells may exhibit different cytokine requirements for growth and survival.

There is some evidence that cytokines are involved in the homeostasis of memory CD8<sup>+</sup> T cells, but evidence for cytokines participating in the homeostasis of naïve CD8<sup>+</sup> T cells is lacking<sup>21</sup>. IL-7 is a cytokine that is involved in the survival and proliferation of thymocytes during early stages of T cell development<sup>22</sup>. *In vitro*, IL-7 increases both the survival and proliferation of mature T cells<sup>23–26</sup> and thus is a potential candidate for a homeostatic cytokine. Because a lack of IL-7 has a dramatic effect on T cell development, the actions of IL-7 on mature T cells *in vivo* have been difficult to test. These previous observations have led us to ask whether IL-7 is involved in the homeostasis of naïve and memory CD8<sup>+</sup> T cells by maintaining T cell survival and inducing proliferation.

## Results IL-7R expression during T cell activation

For analysis of authentic naïve CD8<sup>+</sup> T cells we used OT-I TCR transgenic T cells, which recognize an ovalbumin (OVA) peptide in the context of H-2K<sup>b</sup>. In addition, memory OT-I T cells can be generated *in*

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**Figure 1. IL-7R is regulated during T cell activation and memory induction.** (a) Naive OT-I T cells were identified by gating on CD8<sup>+</sup>CD44<sup>+</sup> cells from LN cells in OT-I RAG<sup>-/-</sup> mice. (b) VSV-OVA-activated and (c) memory OT-I T cells were identified by gating on CD44<sup>hi</sup>Ly-5.2<sup>+</sup> cells from Ly-5.1 host mice that received OT-I T cells (Ly-5.2<sup>+</sup>) and VSV-OVA either 4 days or 2 months previously. (Vertical line, negative control staining.)

*in vivo* by adoptive transfer to Ly-5 congenic hosts followed by immunization<sup>27,28</sup>. Because donor OT-I T cells and the host cells express different Ly-5 isoforms we could analyze or isolate activated and memory CD8<sup>+</sup> T cells directly *ex vivo*. This model system allowed us to examine the expression of IL-7 receptor  $\alpha$  chain (IL-7R) by OT-I T cells during different stages of the immune response.

The IL-7R complex is composed of both the IL-7R chain, which binds IL-7 and thymic stromal lymphopoietin (TSLP)<sup>29</sup>, and the common  $\gamma$  chain that is a shared subunit used by other cytokine receptor complexes<sup>30,31</sup>, but not by the TSLP receptor (TSLPR)<sup>32</sup>. IL-7R expression on naive, activated and memory OT-I T cells was determined by flow cytometry. Naive OT-I RAG<sup>-/-</sup> T cells (Ly-5.2<sup>+</sup>CD8<sup>+</sup>CD44<sup>low</sup>) were transferred to congenic hosts (Ly-5.1<sup>+</sup>), and activated and memory OT-I T cells generated by immunization with vesicular stomatitis virus expressing ovalbumin (VSV-OVA)<sup>33</sup>. Activated and memory OT-I T cells were identified as Ly-5.2<sup>+</sup>CD44<sup>high</sup> cells from mice that were immunized 4 days or 2 months earlier, respectively. IL-7R expression was homogeneously high on all naive and memory OT-I T cells.

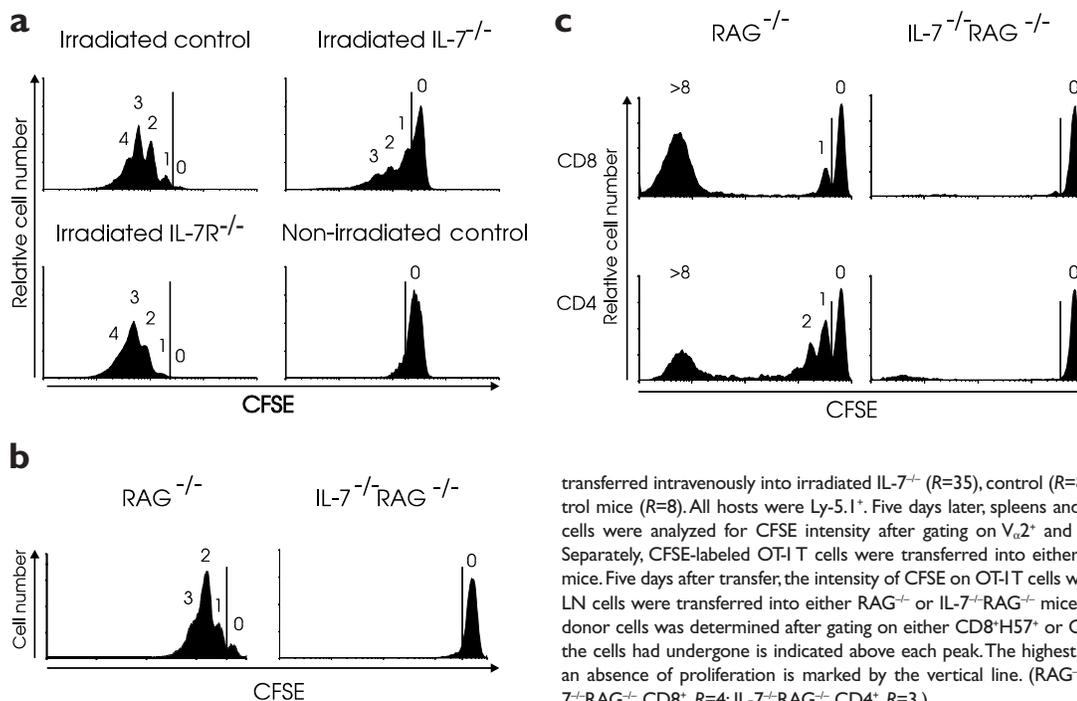
However, most activated OT-I T cells had down-regulated IL-7R with some cells lacking the receptor (Fig. 1). This downmodulation also occurred at the mRNA level (data not shown). These data indicated that IL-7R expression was regulated during T cell differentiation and suggested that naive and memory CD8<sup>+</sup> T cells may be more responsive to IL-7-mediated signaling than recently activated CD8<sup>+</sup> T cells.

### IL-7 and proliferation of naive T cells

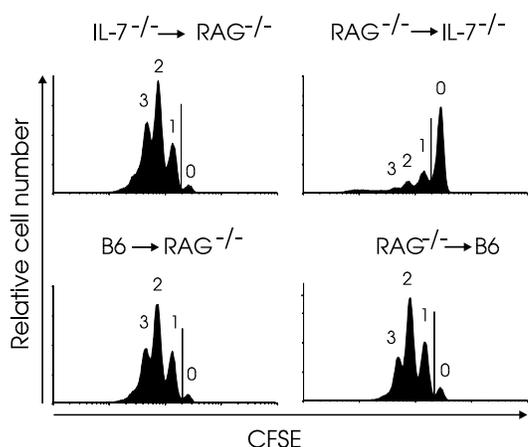
To test whether IL-7R expressed by naive CD8<sup>+</sup> T cells was functional *in vivo*, the effect an absence of IL-7 had on proliferation of OT-I T cells upon transfer into lymphopenic hosts was determined. Lymph node (LN) cells from OT-I mice were labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE) and transferred into sublethally irradiated IL-7<sup>-/-</sup>, irradiated control or nonirradiated control mice. The percentage of the original cell population that had divided (the “responding” population, *R*) was calculated as described<sup>34</sup>. Five days after transfer into irradiated control mice, 88±7% (*n*=5) of the OT-I T cells had proliferated, with the highest percentage of cells having undergone three divisions. Only 35±10% (*n*=5) of OT-I T cells transferred into an irradiated IL-7<sup>-/-</sup> host divided (Fig. 2a).

It was possible that the OT-I T cells had failed to proliferate in the IL-7<sup>-/-</sup> host because the IL-7<sup>-/-</sup> mice displayed abnormal LNs and splenic architecture, primarily as a result of defective B lymphopoiesis. To rule this out, we analyzed the proliferation of OT-I T cells after transfer into irradiated IL-7R<sup>-/-</sup> hosts. IL-7R<sup>-/-</sup> mice have a similar defect in their LNs and splenic architecture as the IL-7<sup>-/-</sup> mice have, but still produce IL-7. Nearly all OT-I T cells transferred into irradiated IL-7R<sup>-/-</sup> hosts divided (*R*=96%, *n*=2) demonstrating that the effect observed in IL-7<sup>-/-</sup> hosts was not simply due to abnormal lymphoid structure (Fig. 2a). OT-I T cells did not proliferate when transferred to a normal, nonirradiated host, or to a nonirradiated IL-7<sup>-/-</sup> host (data not shown).

Because irradiation is known to induce growth factors<sup>35–38</sup>, it was important to test the effect of IL-7 absence without irradiating the host mice. To do so, CFSE-labeled OT-I T cells were transferred into RAG<sup>-/-</sup> or IL-7<sup>-/-</sup>RAG<sup>-/-</sup> mice, both of which lack mature T and B cells. Few, if any, OT-I T cells divided in IL-7<sup>-/-</sup>RAG<sup>-/-</sup> mice (*R*=10±2%, *n*=4) whereas nearly all OT-I T cells (*R*=93±1%, *n*=4) proliferated in RAG<sup>-/-</sup> mice (Fig. 2b). With regard to cell numbers after transfer,



**Figure 2. IL-7 drives the homeostatic proliferation of naive T cells.** (a) CFSE-labeled naive OT-I T cells (Ly-5.2<sup>+</sup>) were transferred intravenously into irradiated IL-7<sup>-/-</sup> (*R*=35), control (*R*=88), IL-7R<sup>-/-</sup> (*R*=97) or unirradiated control mice (*R*=8). All hosts were Ly-5.1<sup>+</sup>. Five days later, spleens and LNs were removed and donor OT-I T cells were analyzed for CFSE intensity after gating on V $\alpha$ 2<sup>+</sup> and Ly-5.2<sup>+</sup> cells using flow cytometry. (b) Separately, CFSE-labeled OT-I T cells were transferred into either RAG<sup>-/-</sup> (*R*=93) or IL-7<sup>-/-</sup>RAG<sup>-/-</sup> (*R*=10) mice. Five days after transfer, the intensity of CFSE on OT-I T cells was determined. (c) CFSE-labeled CD44<sup>+</sup> LN cells were transferred into either RAG<sup>-/-</sup> or IL-7<sup>-/-</sup>RAG<sup>-/-</sup> mice. Six days later, the intensity of CFSE on donor cells was determined after gating on either CD8<sup>+</sup>H57<sup>+</sup> or CD4<sup>+</sup>H57<sup>+</sup> cells. The number of divisions the cells had undergone is indicated above each peak. The highest intensity of CFSE staining, representing an absence of proliferation is marked by the vertical line. (RAG<sup>-/-</sup> CD8<sup>+</sup>, *R*=37; RAG<sup>-/-</sup> CD4<sup>+</sup>, *R*=20; IL-7<sup>-/-</sup>RAG<sup>-/-</sup> CD8<sup>+</sup>, *R*=4; IL-7<sup>-/-</sup>RAG<sup>-/-</sup> CD4<sup>+</sup>, *R*=3.)



**Figure 3. IL-7 produced by non-BM-derived cells is important for CD8<sup>+</sup> T cell proliferation in a lymphopenic host.** BM chimeras were generated and 6 weeks later were irradiated and given CFSE-labeled OT-I T cells. Five days later, OT-I T cells present in the spleen and LN were analyzed for CFSE intensity. The number of cell divisions is indicated above each peak. The highest intensity of CFSE staining, representing an absence of proliferation is marked by the vertical line. (IL-7<sup>-/-</sup>→RAG<sup>-/-</sup>, R = 74; RAG<sup>-/-</sup>→IL-7<sup>-/-</sup>, R = 29; B6→RAG<sup>-/-</sup>, R = 84; RAG<sup>-/-</sup>→B6, R = 85.)

although it is often difficult to obtain reproducible counts in immunodeficient hosts, the values correlated with the *in vivo* results. Thus, larger numbers of cells were present in irradiated IL-7<sup>-/-</sup> mice as compared to IL-7<sup>-/-</sup>RAG<sup>-/-</sup> mice, but in both cases total numbers were less than those obtained from nonirradiated control mice (data not shown). These results implied that survival, as well as proliferation, of transferred cells was IL-7-dependent (see below). Overall, the results indicated that IL-7 was prerequisite for OT-I T cell proliferation in nonirradiated lymphopenic hosts and suggested that irradiation may induce factors affecting proliferation.

To determine whether the homeostatic proliferation of polyclonal T cells has similar requirements for IL-7 as for OT-I T cells, CD44<sup>b6</sup> LN cells from strain- and age-matched normal mice were sorted, CFSE labeled and transferred into either RAG<sup>-/-</sup> or IL-7<sup>-/-</sup>RAG<sup>-/-</sup> hosts. A portion of both CD8<sup>+</sup> and CD4<sup>+</sup> T cells ( $R=37\pm 2\%$ ,  $R=20\pm 2\%$ , respectively) proliferated on transfer into a RAG<sup>-/-</sup> host, in a similar fashion as was reported previously<sup>14</sup>. As with OT-I T cells, polyclonal CD8<sup>+</sup> and CD4<sup>+</sup> T cells failed to proliferate in the IL-7<sup>-/-</sup>RAG<sup>-/-</sup> host ( $R=3\pm 0\%$ ,  $R=3\pm 0\%$ , respectively) (Fig. 2c). These data demonstrated that the requirement for IL-7 in homeostatic proliferation was not unique to the transgenic OT-I T cells but existed for heterogeneous populations of CD8<sup>+</sup> and CD4<sup>+</sup> T cells.

### Homeostasis and radiation-resistant cells

It is thought that IL-7 is primarily produced by stromal cells although there are reports of human dendritic cells producing IL-7<sup>39-42</sup>. We decided to determine the cellular source of the IL-7 acting on T cells. To this end, bone marrow (BM) chimeras were generated in which IL-7<sup>-/-</sup> mice were reconstituted with RAG<sup>-/-</sup> BM and RAG<sup>-/-</sup> mice were reconstituted with IL-7<sup>-/-</sup> BM. As controls, RAG<sup>-/-</sup> mice were reconstituted with C57BL/6 (B6) BM and B6 mice were reconstituted with RAG<sup>-/-</sup> BM. Six weeks later, the chimeras were sublethally irradiated and given CFSE-labeled OT-I T cells. Five days later the majority of the OT-I T cells ( $R=74\pm 14\%$ ,  $n=3$ ) had proliferated in the chimeras generated using IL-7<sup>-/-</sup> BM, as well as in both control chimeras: B6 BM into RAG<sup>-/-</sup> mice ( $R=84\pm 7\%$ ,  $n=4$ ) and RAG<sup>-/-</sup> BM into B6 mice ( $R=85\pm 2\%$ ,  $n=6$ ) (Fig. 3). In contrast, only  $29\pm 14\%$  ( $n=5$ ) of OT-I T cells proliferated in IL-7<sup>-/-</sup> mice that had received RAG<sup>-/-</sup> BM (Fig. 3). These results indicated that radiation-resistant cells, probably non-BM-derived,

were the relevant source of IL-7 for mediating T cell proliferation.

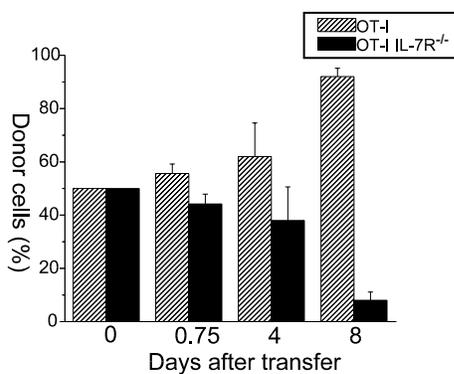
### IL-7 and CD8<sup>+</sup> T cells *in vivo*

Besides inducing proliferation of T cells, IL-7 also maintains the survival of T cells *in vitro*<sup>25,26</sup>. As homeostasis of T cells probably involves survival as well as proliferation, we examined the role of IL-7R in the survival of naïve OT-I T cells. OT-I mice lacking IL-7R were generated. Introducing TCR transgenes into IL-7R-deficient mice allowed the production of mature CD8<sup>+</sup> OT-I T cells, although the total number of cells produced in this strain was ~1–2% of normal, in keeping with the known role of IL-7 in thymocyte development. Nevertheless, sufficient TCR transgenic CD8<sup>+</sup> T cells were obtained for use in adoptive transfer experiments.

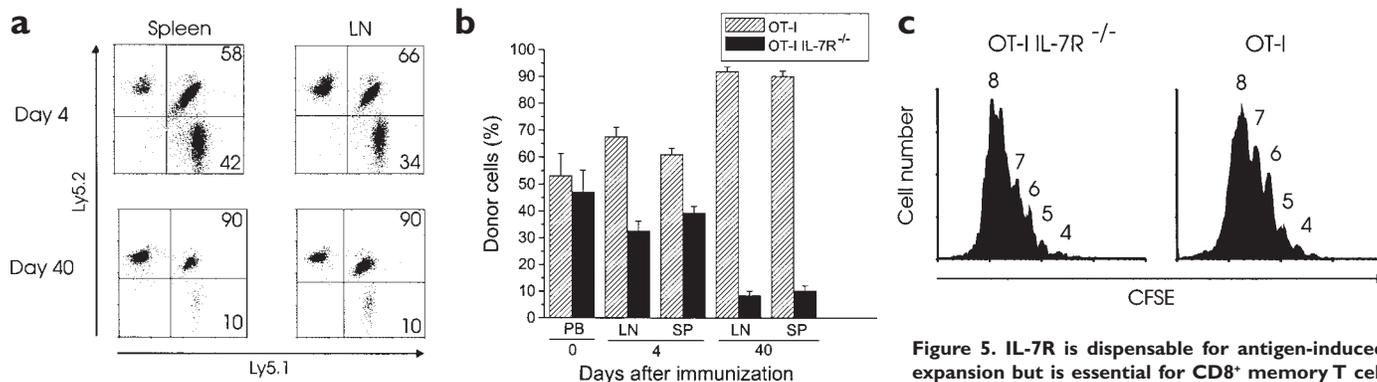
To compare the survival of normal and IL-7R<sup>-/-</sup> OT-I T cells simultaneously, a mixture of OT-I IL-7R<sup>+/+</sup> T cells (Ly-5.1<sup>+</sup>) and OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) were transferred into normal B6 hosts (Ly-5.2<sup>+</sup>). At various times after injection, the ratio of normal OT-I T to IL-7R-deficient OT-I T cells in the peripheral blood or spleen was determined by flow cytometry. Although the overall number of OT-I T cells declined over an 8-day period (data not shown), OT-I IL-7R<sup>-/-</sup> cells were lost at a much greater rate (Fig. 4). On day 8 after transfer, the OT-I IL-7R<sup>-/-</sup> T cells made up <10% of the OT-I population. Thus, IL-7 was required for the survival of mature T cells *in vivo*.

### Activation and memory generation

Considering the pattern of expression of IL-7R on CD8<sup>+</sup> T cells at different activation states, it was of interest to determine the effect of IL-7 on the growth and survival of activated and memory T cells. A mixture containing equal numbers of OT-I and OT-I IL-7R<sup>-/-</sup> cells was transferred to normal mice, which were then infected with VSV-OVA. We examined the ratio of OT-I to OT-I IL-7R<sup>-/-</sup> cells at the peak of the primary response 4 days after infection. Substantial expansion of normal and OT-I IL-7R<sup>-/-</sup> T cells had occurred from 0.2% of CD8<sup>+</sup> T cells before infection to 16% and 40% of CD8<sup>+</sup> T cells in LN and spleen, respectively, on day 4 after infection. At this time, the ratio of normal OT-I T to OT-I IL-7R<sup>-/-</sup> cells was ~60:40 in the spleen and LNs indicating that the OT-I IL-7R<sup>-/-</sup> cells had greatly expanded in response to the virus infection (Fig. 5a). In contrast, 40 days after infection the OT-I memory population was made up primarily of normal cells due to the loss of OT-I IL-7R<sup>-/-</sup> cells (Fig. 5a,b). Six hours after transfer of the mixture, OT-I IL-7R<sup>-/-</sup> T cells made up  $47\pm 8\%$  ( $n=8$ ) of the total OT-I



**Figure 4. IL-7R is required for CD8<sup>+</sup> T cell survival in normal hosts.** OT-I IL-7R<sup>-/-</sup> T cells (Ly-5.1<sup>+</sup>) and normal OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) were mixed in equal proportions and injected into normal B6 (Ly-5.2<sup>+</sup>) hosts. The proportion of each donor cell population was determined in the injection mixture (at day=0), in peripheral blood lymphocytes after 18 h (0.75 days) and 4 days, and in the spleen after 8 days. Cells were gated based on Ly-5.1, Ly-5.2, V $\alpha$ 2 and CD8<sup>+</sup> expression. The graph depicts the ratio of OT-I to OT-I IL-7R<sup>-/-</sup> cells at the indicated times after transfer. Values are the mean of four animals  $\pm$  s.d.



**Figure 5. IL-7R is dispensable for antigen-induced expansion but is essential for CD8<sup>+</sup> memory T cell production.** (a) Representative flow cytometric profiles showing the ratio of OT-I (Ly-5.1<sup>+</sup>Ly-5.2<sup>-</sup>) to OT-I IL-7R<sup>-/-</sup> (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) cells were injected into normal B6 (Ly-5.2<sup>+</sup>) hosts, which were then immediately immunized with VSV-OVA. The proportion of each donor cell population was determined in the peripheral blood 6 h after transfer (day=0), at the peak of the response (day 4) and during the memory phase (day 40). V<sub>2</sub>, CD8<sup>+</sup> cells were gated and analyzed for Ly-5.1 and Ly-5.2 expression. Values are the percentages of OT-I or OT-I IL-7R<sup>-/-</sup> cells in the donor population. (b) Average ratio of OT-I to OT-I IL-7R<sup>-/-</sup> cells present in peripheral blood (PB), lymph nodes (LN) and spleen (SP) at various time points after transfer. Ratios are the mean±s.d. from four to eight animals. (c) Mixtures of OT-I IL-7R<sup>-/-</sup> T cells (Ly-5.1<sup>+</sup>) and normal OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) were labeled with CFSE and injected into normal mice which were then immediately infected with VSV-OVA. Three days after immunization, both OT-I T cell populations present in the LNs were analyzed for CFSE intensity. The number of cell divisions undergone is indicated above each peak.

(Ly-5.1<sup>+</sup>) T cells 4 or 40 days after immunization. Mixtures of OT-I IL-7R<sup>-/-</sup> T cells (Ly-5.1<sup>+</sup>) and normal OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) were injected into normal B6 (Ly-5.2<sup>+</sup>) hosts, which were then immediately immunized with VSV-OVA. The proportion of each donor cell population was determined in the peripheral blood 6 h after transfer (day=0), at the peak of the response (day 4) and during the memory phase (day 40). V<sub>2</sub>, CD8<sup>+</sup> cells were gated and analyzed for Ly-5.1 and Ly-5.2 expression. Values are the percentages of OT-I or OT-I IL-7R<sup>-/-</sup> cells in the donor population. (b) Average ratio of OT-I to OT-I IL-7R<sup>-/-</sup> cells present in peripheral blood (PB), lymph nodes (LN) and spleen (SP) at various time points after transfer. Ratios are the mean±s.d. from four to eight animals. (c) Mixtures of OT-I IL-7R<sup>-/-</sup> T cells (Ly-5.1<sup>+</sup>) and normal OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) were labeled with CFSE and injected into normal mice which were then immediately infected with VSV-OVA. Three days after immunization, both OT-I T cell populations present in the LNs were analyzed for CFSE intensity. The number of cell divisions undergone is indicated above each peak.

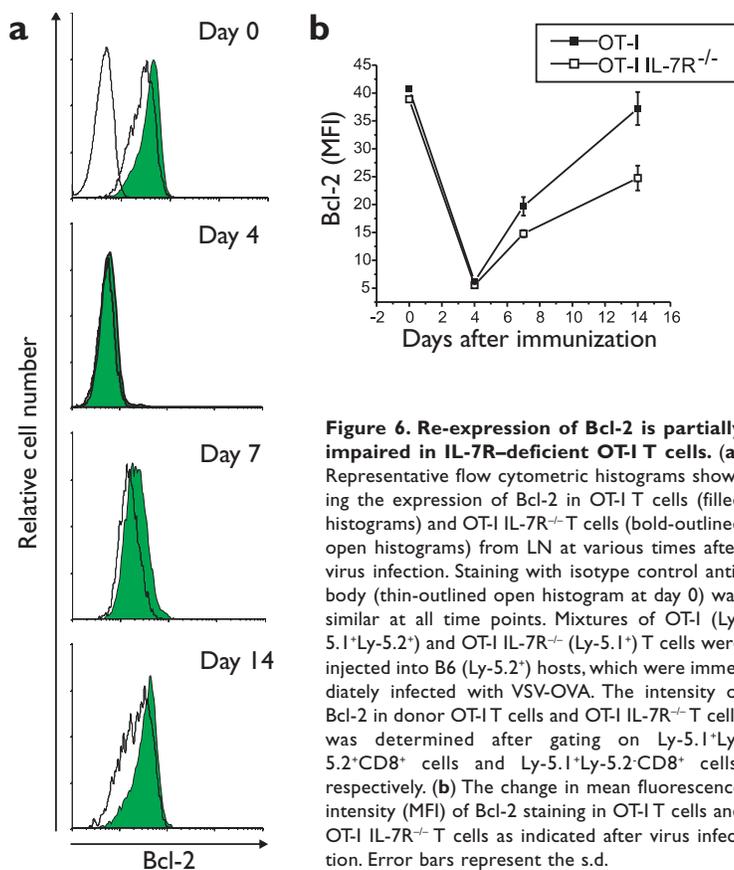
T cell population in the peripheral blood. Four days after immunization, OT-I IL-7R<sup>-/-</sup> cells made up 34±2% (n=4) and 42±2% (n=4) of the total OT-I T cell population in LNs and spleen respectively (Fig. 5a,b). Forty days after immunization, OT-I IL-7R<sup>-/-</sup> T cells made up only 10±2% (n=4) and 8±2% (n=4) of the donor OT-I T cells in the spleen and LNs, respectively (Fig. 5b). This data demonstrated that IL-7R-deficient OT-I T cells were less likely to survive and differentiate into memory T cells.

The relative decrease in the number of OT-I IL-7R<sup>-/-</sup> T cells at day 4 after infection could have been due to decreased ability to respond to the infection or to a decreased survival of OT-I IL-7R<sup>-/-</sup> T cells. Therefore, the experiment was repeated in a similar manner except that the OT-I T cells were labeled with CFSE before transfer and an earlier time point was analyzed. Three days after infection with VSV-OVA, most of the OT-I IL-7R<sup>-/-</sup> T cells and the normal OT-I T cells had undergone more than seven divisions in the spleen (data not shown). In the peripheral LNs, proliferation of the OT-I T and OT-I IL-7R<sup>-/-</sup> T cells had lagged behind that detected in the spleen, with the cells having undergone four to eight divisions (Fig. 5c). In both tissues the proliferation pattern of the OT-I IL-7R<sup>-/-</sup> T cells was the same as in normal OT-I T cells (Fig. 5c). These results suggested that the decrease in the ratio of mutant to control T cells early during the immune response was due to lack of IL-7, or perhaps TSLP-mediated survival signals.

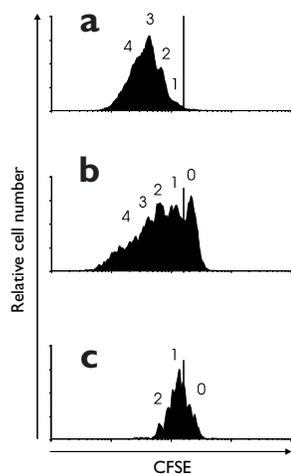
### IL-7 and regulation of Bcl-2

Relative to Bcl-2 expression in naïve CD8<sup>+</sup> T cells, Bcl-2 decreases in CD8<sup>+</sup> T cells at the peak of proliferation in response to a virus infection and is expressed at higher levels by memory CD8<sup>+</sup> T cells<sup>43</sup>. As IL-7 regulates Bcl-2<sup>44-46</sup>, we determined whether regulation of Bcl-2 during an immune response required IL-7. Before injection and at various times after immunization, Bcl-2 expression was compared in OT-I IL-7R<sup>-/-</sup> T cells and normal OT-I T cells. Bcl-2 expression in naïve OT-I IL-7R<sup>-/-</sup> T cells and OT-I T cells was similar before transfer (Fig. 6a,b). Mixtures of OT-I IL-7R<sup>-/-</sup> T cells (Ly-5.1<sup>+</sup>) and OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) were transferred into normal B6 hosts (Ly-5.2<sup>+</sup>),

which were then immunized with VSV-OVA. Four days after immunization, expression of Bcl-2 in the OT-I IL-7R<sup>-/-</sup> T cells and OT-I T cells decreased to levels comparable to staining with isotype control antibody (Fig. 6a,b). In OT-I T cells, Bcl-2 expression gradually increased 4–14 days after immunization, eventually returning to naïve CD8<sup>+</sup> T cell levels (Fig. 6a,b). In contrast, the up-regulation of Bcl-2 in OT-I IL-7R<sup>-/-</sup> T cells lagged behind that of normal OT-I T cells, result-



**Figure 6. Re-expression of Bcl-2 is partially impaired in IL-7R-deficient OT-I T cells.** (a) Representative flow cytometric histograms showing the expression of Bcl-2 in OT-I T cells (filled histograms) and OT-I IL-7R<sup>-/-</sup> T cells (bold-outlined open histograms) from LN at various times after virus infection. Staining with isotype control antibody (thin-outlined open histogram at day 0) was similar at all time points. Mixtures of OT-I (Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>) and OT-I IL-7R<sup>-/-</sup> (Ly-5.1<sup>+</sup>) T cells were injected into B6 (Ly-5.2<sup>+</sup>) hosts, which were immediately infected with VSV-OVA. The intensity of Bcl-2 in donor OT-I T cells and OT-I IL-7R<sup>-/-</sup> T cells was determined after gating on Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>CD8<sup>+</sup> cells and Ly-5.1<sup>+</sup>Ly-5.2<sup>+</sup>CD8<sup>+</sup> cells, respectively. (b) The change in mean fluorescence intensity (MFI) of Bcl-2 staining in OT-I T cells and OT-I IL-7R<sup>-/-</sup> T cells as indicated after virus infection. Error bars represent the s.d.



**Figure 7. Memory CD8<sup>+</sup> OT-I T cells are partially dependent on IL-7 for homeostatic proliferation.** Ly-5.2 enriched, memory OT-I T cells were CFSE-labeled and transferred into either irradiated control (R=94), irradiated IL-7<sup>-/-</sup> (R=61) or nonirradiated (R=40) control mice. After 5 days, the number of cell divisions was determined by analyzing the CFSE intensity of Ly-5.2<sup>+</sup>V $\alpha$ 2<sup>+</sup> cells. The highest intensity of CFSE staining, representing an absence of proliferation, is marked by the vertical line.

ing in an overall lower level of Bcl-2 expression at day 14 (Fig. 6a,b). These data demonstrated that down-regulation of Bcl-2 was IL-7-independent but that re-expression of Bcl-2 was partially impaired in the

absence of IL-7. This suggested that lower Bcl-2 expression may be responsible for the greater decline in OT-I IL-7R<sup>-/-</sup> T cells compared to normal OT-I T cells.

### Proliferation of memory CD8<sup>+</sup> T cells

Because CD8<sup>+</sup> T cell memory generation and/or maintenance was IL-7-dependent, we determined whether homeostatic proliferation of memory cells required IL-7. Memory OT-I T cells (Ly-5.2<sup>+</sup>) were purified from the spleen and LNs of adoptively transferred VSV-OVA-infected mice, CFSE-labeled and transferred to irradiated IL-7<sup>-/-</sup> or irradiated control mice, or to nonirradiated control mice (all Ly-5.1<sup>+</sup>). After five days, 94±3% (n=4) of the memory OT-I T cells had divided in the irradiated control mice (Fig. 7). In the irradiated IL-7<sup>-/-</sup> host, 61±14% (n=3) of the total population had divided. This result indicated that IL-7 was important for the induction of proliferation in a subset of memory cells in irradiated hosts. In addition, the population that had divided showed a delayed onset of division as compared to controls (Fig. 7). Unlike naïve OT-I T cells, 40% of memory OT-I T cells (mean of two samples) divided once or twice after transfer to non-irradiated hosts, in agreement with previous reports<sup>9,10,16,17</sup>.

## Discussion

Although recent work has pointed to an obligatory role for MHC molecules in maintaining homeostasis of the naïve peripheral CD8<sup>+</sup> and CD4<sup>+</sup> T cell pools, it is not known whether cytokines are also involved in this process. The reason why IL-7R is expressed by most naïve CD8<sup>+</sup> T cells is also unclear. Our results indicated that, in the case of naïve CD8<sup>+</sup> and CD4<sup>+</sup> T cells, IL-7 was required for induction of homeostatic proliferation in lymphopenic hosts. For CD8 T cells, IL-7 was also essential for survival in normal hosts, but was not needed for expansion of CD8<sup>+</sup> T cells responding to a virus infection. This disagrees with *in vitro* studies showing that IL-7R<sup>-/-</sup> T cells proliferated poorly in response to phorbol 12-myristate 13-acetate and ionomycin or a monoclonal antibody to CD3<sup>24</sup>, suggesting that additional growth-promoting factors may be available *in vivo*.

Our findings correlated with the constitutive expression of IL-7R on naïve CD8<sup>+</sup> T cells and the down-regulation of IL-7R after activation. Thus, IL-7 is not only critical for normal lymphocyte development in BM and thymus<sup>22,47</sup> but is essential for overall maintenance of peripheral CD8<sup>+</sup> T cell survival. For naïve CD4 T cell survival, two  $\gamma$ -utilizing cytokines, IL-4 and IL-7, are required<sup>19</sup>. This finding is supported by the demonstration that TCR transgenic CD4 thymocytes require  $\gamma$

receptors for survival after transfer to alymphoid hosts<sup>20</sup>. Our results show that the relevant cytokine for homeostatic proliferation of naïve CD4<sup>+</sup> T cells is IL-7.

The molecular mechanism by which IL-7 regulates T cell survival may involve Bcl-2. Bcl-2 is an antiapoptotic protein that becomes up-regulated once thymocytes have matured, decreases during T cell activation and is again up-regulated in memory T cells<sup>45,46</sup>. Bcl-2, therefore, is implicated in maintaining the survival of naïve and memory T cells. Prevention of apoptosis by IL-7 *in vitro* is accompanied by an increase in Bcl-2<sup>25</sup>. Nevertheless, Bcl-2 expression was not decreased on naïve IL-7R-deficient OT-I T cells. This is in contrast to  $\gamma$ -deficient CD4<sup>+</sup> T cells, which have decreased Bcl-2 expression and decreased survival compared to normal CD4<sup>+</sup> T cells<sup>20</sup>. Thus, CD4<sup>+</sup> T cells may require other  $\gamma$  cytokines for maintaining Bcl-2 expression.

Because naïve IL-7R-deficient OT-I cells expressed normal levels of Bcl-2, their decreased survival was probably not due to dysregulated Bcl-2 levels but could be due to a decreased expression of other IL-7-regulated survival factors, such as the transcription factor Lung Kruppel-Like Factor (LKLf). Mice lacking LKLf harbor naïve T cells with a shorter lifespan<sup>48</sup>. LKLf, like IL-7R, is down-regulated upon activation but then re-expressed by memory T cells<sup>28</sup>. Treatment of activated T cells with IL-7 *in vitro* increases the expression of LKLf, which correlates with increased T cell survival and the acquisition of a memory T cell phenotype<sup>28</sup>. Overall, IL-7 may promote survival of naïve T cells or prevent apoptosis of activated T cells by regulating LKLf and other factors and we are currently assessing this possibility.

Because IL-7 increases T cell survival, one could speculate that the downmodulation of IL-7R is important for promoting T cell death after activation, thus promoting contraction of the immune response and generation of a controlled number of memory cells. This hypothesis is supported by the down-regulation of IL-7R coinciding with the down-regulation of Bcl-2. However, the down-regulation of Bcl-2 during an immune response is functional in cells lacking IL-7R. In light of this finding, we speculate that the re-expression of IL-7R participates in the up-regulation of Bcl-2 during and/or subsequent to contraction of the immune response. Cell death after an immune response is important for re-establishing homeostasis, a limited number of antigen-activated T cells survive and differentiate into memory T cells expressing IL-7R and Bcl-2.

We demonstrated that up-regulation of Bcl-2 is not completely dependent on IL-7 because the IL-7R-deficient T cells present after the peak of the immune response expressed Bcl-2, though at lower levels as a population as a whole. It should be noted, however, that only surviving cells could be analyzed, so that those cells that may have failed to up-regulate Bcl-2 levels and therefore died were not part of our analysis. IL-7R expression by normal memory cells may either have been the result of re-expression after down-regulation or may have been due to the retention of IL-7R by a subset of activated T cells. In either case, the expression of IL-7R at the memory cell stage promoted survival. This was supported by our demonstration that OT-I IL-7R<sup>-/-</sup> cells responded well to a virus infection but were ineffective at producing memory cells.

Once memory was established in normal hosts, we showed a partial requirement for IL-7 in mediating memory cell homeostasis. The incomplete block of memory T cell proliferation in the absence of IL-7 suggested that other factors may be involved in maintaining CD8<sup>+</sup> T cell memory. Memory CD8<sup>+</sup> T cells also express the IL-15R complex, which can mediate proliferation and survival in a normal host<sup>18,49,50</sup>, and mice lacking IL-15R or IL-15 are deficient in memory phenotype CD8<sup>+</sup> T cells<sup>50,51</sup>. It is not known whether IL-15 mediates proliferation of mem-

ory CD8<sup>+</sup> T cells in a lymphopenic host or whether IL-7 and IL-15 can act in concert. It was demonstrated that blocking IL-7 was inconsistent in inhibiting proliferation of memory CD8 T cells in a normal host<sup>18</sup> suggesting that the mechanisms mediating homeostatic proliferation may be more dependent on IL-7 than the mechanisms maintaining the continual low-level proliferation of memory T cells that occurs in a normal host. In addition, experiments where IL-7 blocking was effective were those using younger mice, which could suggest a temporal requirement for IL-7 in memory T cell proliferation<sup>18</sup>. Unlike naïve cells, at least some memory T cells undergo continual low-level proliferation suggesting that division and survival are independently regulated. Under normal conditions IL-15 may participate in regulating division whereas IL-7 could be essential for survival.

The cell type involved in IL-7-mediated homeostasis is unknown but our results point to a radiation-resistant cell, perhaps a non-BM-derived stromal cell. This is in line with the known expression pattern of IL-7 in thymic and intestinal epithelial cells as well as BM stroma<sup>39,52,53</sup>. Studies have demonstrated that MHC class I and II are needed for naïve CD8<sup>+</sup> and CD4<sup>+</sup> T cell survival and homeostatic proliferation indicating that cell-cell contact is required<sup>13–15,54</sup>. Whereas MHC class I is expressed on all nucleated cells, a non-BM-derived stromal cell could provide the necessary MHC interaction as well as IL-7 for CD8<sup>+</sup> T cell homeostasis. However, CD4<sup>+</sup> T cells require MHC class II for survival and homeostatic proliferation suggesting that BM-derived cells are responsible for the relevant interaction.

Dendritic cells expressing MHC class II are required for survival of CD4<sup>+</sup> T cells<sup>7</sup> but it is unknown whether this cell type is involved in homeostatic proliferation. Thus either CD8<sup>+</sup> and CD4<sup>+</sup> T cells are maintained by different cell types, or the same cell type is involved in the MHC interaction and IL-7 is acquired from another source. IL-7 can be found associated with the extracellular matrix<sup>55</sup>, which provides a possible mechanism by which IL-7 may be obtained by T cells during their migration through lymphoid organs. Our results provided strong evidence for an interaction between hematopoietic cells and a stroma-derived factor that was essential for the control of naïve T cell survival and memory T cell generation.

## Methods

**Mice.** C57BL/6J and C57BL/6-IL-7R $\alpha$ <sup>-/-</sup> mice<sup>56</sup> were obtained from Jackson Laboratories (Bar Harbor, ME). C57BL/6-Ly-5.2 mice were obtained from Charles River (Wilmington, MA) through the National Cancer Institute. The OT-I mouse line<sup>57</sup> was provided by W. R. Heath (Walter and Eliza Hall Institute, Parkville, Australia) and F. Carbone (Monash Medical School, Prahran, Victoria, Australia) and was maintained on a C57BL/6-RAG<sup>-/-</sup> background (either Ly-5.1<sup>+</sup>Ly-5.2<sup>-</sup> or Ly-5.1<sup>+</sup>Ly-5.2<sup>-</sup>). C57BL/6/129Ola IL-7<sup>-/-</sup> mice<sup>22</sup> were originally obtained from R. Murray and U. von Freeden-Jeffry (DNAX). IL-7<sup>-/-</sup>RAG-2<sup>-/-</sup> (ref. 22) and age- and background-matched RAG-2<sup>-/-</sup> mice were provided by D. Rennick (DNAX) through Taconic Farms. All mice were maintained under specific pathogen-free conditions. Mice were handled under protocols approved by the UCHC Animal Care Committee according to federal guidelines.

**Flow cytometric analysis.** Lymphocytes were resuspended in PBS, bovine serum albumin (0.2%) and NaN<sub>3</sub> (0.1%) at a concentration of 1–10 $\times$ 10<sup>6</sup> cells/ml, followed by incubation of cells (100  $\mu$ l) at 4 °C for 20 min with 100  $\mu$ l of properly diluted monoclonal antibody. Monoclonal antibodies specific for the following antigens and coupled to the indicated fluorochromes were used: fluorescein isothiocyanate (FITC)-V $\beta$ 5, phycoerythrin (PE)-V $\alpha$ 2, PE-CD44, peridini chlorophyll protein (PerCP)-CD8 PE-CD4, allophycocyanin-H57 (all from PharMingen, San Diego, CA); FITC(3.168)-CD8 $\alpha$  or biotin-CD8 $\alpha$ <sup>58</sup>; and FITC- or Cy5-conjugated Ly-5.1- or Ly-5.2<sup>59</sup>. Streptavidin-PE-Cy7 (Caltag Laboratories, Burlingame, CA) and streptavidin-Cy5 (Jackson ImmunoResearch, West Grove, PA) were used to detect biotinylated monoclonal antibodies. Relative fluorescence intensities were measured with a FACScalibur (Becton Dickinson, San Jose, CA). Data were analyzed using WinMDI software (Joseph Trotter, Scripps Clinic, La Jolla, CA).

To detect cell surface expression of IL-7R $\alpha$ , cells were incubated with mouse IgG (200  $\mu$ g/ml) to block nonspecific binding, then reacted with culture supernatant containing anti-IL-7R $\alpha$  (A7R34, IgG2b)<sup>60</sup> or control rat IgG2b (PharMingen) followed by biotinylated rabbit anti-rat and streptavidin-Cy5 (Jackson ImmunoResearch). Samples were blocked

with a mixture of rat and hamster immunoglobulin (200  $\mu$ g/ml) to saturate free immunoglobulin binding sites before staining with FITC- and PE-conjugated antibodies specific for the antigens of interest. Anti-IL-7R $\alpha$  was a gift of S. -I. Nishikawa (Kyoto University, Japan).

Before staining for intracellular Bcl-2, cells (2 $\times$ 10<sup>6</sup>) were stained for cell surface antigens as described above. After washing, cells were fixed and permeabilized with Cytofix–Cytoperm solution according to manufacturer's instructions (PharMingen). Cells were stained with either FITC-conjugated hamster anti-mouse Bcl-2 (clone 3F11) or an isotype FITC-conjugated control antibody to hamster (PharMingen) diluted in perm/wash buffer.

**CFSE labeling and adoptive transfer.** LNs from OT-I mice was homogenized in HBSS with HEPES, L-glutamine, penicillin, streptomycin, gentamycin sulfate (HBSS-HGPG) and filtered through NITEX nylon mesh (Tetko, Kansas City, MO). To determine the percentage of transgenic OT-I T cells present, cells were stained for V $\alpha$ 2, V $\beta$ 5, and CD8<sup>+</sup>. LN cells were resuspended in HBSS-HGPG (10 $\times$ 10<sup>6</sup> cells/ml) and warmed to 37 °C, they were then incubated for 10 min with 5-,6-CFSE (0.01mM, Molecular Probes, Eugene, OR) followed by two washes with HBSS-HGPG. The cells were analyzed by flow cytometry and the intensity of CFSE staining varied between experiments. CFSE-labeled cells (1–5 $\times$ 10<sup>6</sup>) were resuspended in PBS and adoptively transferred into various hosts by intravenous (i.v.) injection. The percentage of cells of the original population that had divided (R) was calculated as described<sup>34</sup>.

For analysis of polyclonal T cells, LNs from strain- and age-matched 129SVE mice were homogenized and stained with PE-anti-CD44. CD44<sup>lo</sup> cells were sorted by a FACStar Plus (Becton-Dickinson), labeled with CFSE, and transferred i.v. into either 129SVE RAG<sup>-/-</sup> or 129SVE IL-7<sup>-/-</sup>RAG<sup>-/-</sup> hosts. Six days later, LNs and spleen were collected and the intensity of CFSE staining on donor cells determined after gating on either CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> or CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> cells.

To generate lymphopenic hosts, mice were  $\gamma$ -irradiated (700 rad) from a <sup>137</sup>Cs source. CFSE-labeled cells were injected within 24 h of irradiation. BM chimeras were generated by lethal irradiation of host mice (1100 rad) before i.v. injection of 5 $\times$ 10<sup>6</sup> BM cells. BM cells were obtained from femurs and tibias of IL-7<sup>-/-</sup>, RAG-1<sup>-/-</sup> or C57BL/6 mice. Six weeks later the mice were irradiated (700 rad) to generate a lymphopenic environment and then given 5 $\times$ 10<sup>6</sup> CFSE-labeled OT-I T cells.

**Adoptive transfer of OT-I and OT-I IL-7R<sup>-/-</sup> cell mixtures and virus infection.** A mixture containing an equal number (2 $\times$ 10<sup>6</sup> total cells) of OT-I T cells (Ly-5.1<sup>+</sup>Ly-5.2<sup>-</sup>) and OT-I IL-7R<sup>-/-</sup> cells (Ly-5.1) from spleen and LN was injected i.v. into normal C57BL/6-Ly-5.2 hosts. The mixtures were analyzed to determine the actual ratio of each set of donor cells by staining with monoclonal antibodies to Ly-5.1, Ly-5.2, V $\alpha$ 2 and CD8. Within 3 h of receiving T cell transfer, mice were infected intravenously with 1 $\times$ 10<sup>6</sup> plaque-forming units (PFU) of recombinant VSV-OVA<sup>33</sup>. At various time points afterwards, peripheral blood and LN or splenic lymphocytes were isolated and analyzed for expression of Ly-5.1, Ly-5.2, V $\alpha$ 2 and CD8.

**Enrichment of memory OT-I T cells.** Memory OT-I T cells were generated by adoptively transferring LN cells from naïve Ly-5.2 OT-I mice into C56BL/6-Ly-5.1 mice and immunizing with 1 $\times$ 10<sup>6</sup> PFU of VSV-OVA. At least 2 months later, cells from spleen and LN were incubated with biotinylated anti-Ly-5.1 for 20 min at 4 °C. The cells (10<sup>8</sup> cells/ml) were washed twice in PBS and bovine serum albumin and then incubated with Streptavidin-Microbeads (1:10 dilution, Miltenyi Biotech, Germany) for 15 min at 4 °C. Donor, Ly-5.2<sup>-</sup> cells were enriched by negative selection using CS<sup>+</sup> depletion columns in conjunction with a VarioMACS magnet according to manufacturers instructions (Miltenyi Biotech.). After enrichment, memory OT-I T cells comprised 70–80% of the population.

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