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Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells

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Abstract

Although interleukin-2 (IL-2) was initially characterized as the primary T-cell growth factor following *in vitro* activation1, less is known about its role in shaping T-cell responses to acute infections *in vivo*. The use of IL-2- or IL-2-receptor-deficient mice is problematic owing to their early development of autoimmunity2–5, attributable to the central role of IL-2 in the generation, maintenance and function of $CD4^+CD25^+$ regulatory T cells6–9. To bypass these inherent difficulties, we have studied the effect of IL-2 on T-cell responses to acute infections by adopting a mixed chimaera strategy in which T cells lacking the high-affinity IL-2 receptor could be studied in an otherwise healthy mouse containing a full complement of regulatory T cells. Here we show that although IL-2 signalling to pathogen-specific $CD8^+$ T cells affects the number of developing effector and memory cells very little, it is required for the generation of robust secondary responses. This is not due to an altered T-cell-receptor repertoire development or selection, and does not reflect an acute requirement for IL-2 during secondary activation and expansion. Rather, we demonstrate a previously unappreciated role for IL-2 during primary infection in programming the development of $CD8^+$ memory T cells capable of full secondary expansion. These results have important implications for the development of vaccination or immunotherapeutic strategies aimed at boosting memory T-cell function.

In recent years, many factors required for the generation, homeostatic turnover, and long-term survival of memory T cells have been identified, including cytokines such as IL-7 and IL-15 (refs 10–13). $CD4^+$ T cells, though largely dispensable for primary $CD8^+$ T cell responses to many acute infections, play a particularly crucial role in the generation of functional $CD8^+$ memory T cells14–18. IL-2, a product of $CD4^+$ T cells, is a member of the same cytokine family as IL-7 and IL-15 by virtue of its shared usage of the common γ chain as part of its receptor and was an obvious candidate for further study. Early reports conflict on the ability of T cells to respond to infection in the setting of IL-2 deficiency19,20, but these studies remain difficult to interpret, because IL-2- and IL-2R-deficient mice develop lymphoproliferation and autoimmunity at an early age2–5. More recent attempts to study IL-2 in healthy hosts have found that it plays a surprisingly modest role in driving the development of effector cytotoxic T lymphocytes21–23.

We aimed to study the long-term impact of IL-2 signalling on the development of $CD8^+$ memory T-cell numbers and function in mice that did not suffer from ongoing autoimmune disease. To create this setting, we made mixed bone-marrow chimaeras in which irradiated

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C57BL/6 (B6) mice (CD45.1$^+$) were reconstituted with T-cell-depleted bone marrow from B6 (Thy1.1$^+$) and IL-2Rα-deficient (Thy1.2$^+$) donors (hereafter referred to as WT/*IL-2Rα*$^{-/-}$ chimaeras), thus allowing us to track wild-type (WT) and IL-2Rα-deficient T-cell responses in a healthy mouse with a full complement of regulatory T cells (Supplementary Fig. 1).

Following acute infection of WT/*IL-2Rα*$^{-/-}$ chimaeras with lymphocytic choriomeningitis virus (LCMV), both WT and IL-2Rα-deficient CD8$^+$ T cells generated comparable populations of effector and memory cells specific for the immunodominant epitope of the LCMV glycoprotein, $GP_{33–41}$ (Fig. 1a). Similar results were seen for another immunodominant CD8 epitope, $NP_{396–404}$, and for the immunodominant CD4 epitope $GP_{61–80}$ (data not shown). However, following a rechallenge with *Listeria monocytogenes* secreting the $GP_{33–41}$ epitope (LM-GP), IL-2Rα-deficient memory cells mounted highly impaired secondary responses (Fig. 1a). Whereas the frequencies of WT to IL-2Rα-deficient memory cells were maintained at or near a ratio of 2:1, this ratio rose to 14:1 after re-challenge (Fig. 1b). Furthermore, while WT memory cells expanded nearly 40-fold after re-challenge, IL-2Rα-deficient memory cells only expanded fourfold (Fig. 1c). These results were repeated when LCMV-immune chimaeras were re-challenged with a high dose of LCMV (Supplementary Fig. 2) and when chimaeras immune to a recombinant *Listeria monocytogenes* secreting ovalbumin (LM-Ova) were given a high-dose secondary challenge with LM-Ova (Supplementary Fig. 3).

To determine whether the inability of IL-2Rα-deficient memory cells to respond to re-challenge was due to altered T-cell repertoire development or selection in the absence of IL-2 signalling, we measured the responses of WT and IL-2Rα-deficient P14 T-cell-receptor transgenic cells (specific for the $GP_{33–41}$ epitope of LCMV) after LCMV infection. To ensure that these cells would develop in hosts with sufficient regulatory T cells, we generated mixed chimaeras using a mixture of B6 bone marrow and either WT or IL-2Rα-deficient P14 bone marrow to reconstitute lethally irradiated B6 mice. Eight weeks later, CD44^{lo} P14 T cells were harvested from these chimaeras and co-transferred into new B6 hosts, followed by LCMV infection (Supplementary Fig. 4). Because the WT P14 donors were Thy1.2$^+$, the IL-2Rα-deficient P14 donors were Thy1.1$^+$/1.2$^+$, and the recipient host was Thy1.1$^+$, we were able to measure the responses of WT and IL-2Rα-deficient P14 cells in the same host.

Following LCMV infection, both WT and IL-2Rα-deficient P14 T cells expanded robustly and developed long-term memory populations in the spleen (Fig. 2a, b) and in the mesenteric lymph nodes and liver (Supplementary Fig. 5). Furthermore, both WT and IL-2Rα-deficient P14 memory cells displayed normal homeostatic turnover, as assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation over a 7-day period (Fig. 2c). Both groups of memory cells expressed similarly high levels of CD122 and CD44 (data not shown), while the frequency of cells expressing CD62L and IL-7Rα was actually higher at all memory time points in the IL-2Rα-deficient population than in the WT memory population (Fig. 2d). Furthermore, following *ex vivo* re-stimulation IL-2Rα-deficient memory cells rapidly upregulated CD69 and contained a consistently higher frequency of IL-2-producers than WT memory cells (Supplementary Fig. 6). The phenotype of the IL-2Rα-deficient cells was consistent with a central memory-like phenotype, which has been shown to correlate with high proliferative and protective capacity24. The rapid loss of CD62L^{lo} cells in the IL-2Rα-deficient population may reflect a previously suggested role for IL-2 in driving the differentiation of effector memory25. A recent study observed that the early accumulation of cells with a central memory phenotype depends on a high precursor frequency of antigen-specific cells26. We found that CD62L^{hi} cells emerged more rapidly in the IL-2Rα-deficient population even when as few as 500 WT and IL-2Rα-deficient P14 cells were co-transferred so that precursor frequencies approached endogenous levels (Supplementary Fig. 7).

At memory time points, WT and IL-2Rα-deficient P14 cells were sorted and transferred into new B6 hosts in equal numbers. Following a secondary LCMV challenge, the WT memory cells far outpaced the IL-2Rα-deficient memory cells in their ability to expand (Fig. 3a). Whereas WT P14 T cells had only a 2:1 advantage over the IL-2Rα-deficient P14 T cells at the peak of the primary effector response, at the peak of the secondary response this ratio approached 20:1 (Fig. 3b). Similar results were again obtained when the precursor frequency of transferred P14 cells was similar to endogenous precursor frequencies (Supplementary Fig. 7), showing that IL-2 plays a central role for long-term protective immunity independently of T-cell repertoire development or selection. Following co-transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled WT and IL-2Rα-deficient memory P14 into new hosts and re-challenge with LCMV, both groups rapidly diluted their CFSE within three days. However, even within this short time of antigen re-exposure WT P14 cells accumulated to far greater numbers than IL-2Rα-deficient P14 cells (Fig. 3c), indicating that IL-2 is not required to drive proliferation of memory cells following secondary antigen encounter but instead promotes the survival and accumulation of dividing cells.

We hypothesized that IL-2 signalling could play a role in promoting secondary T-cell expansion during three separate phases of the immune response: during the primary response, during memory maintenance, or following re-challenge. To address this question, we took advantage of the recent observation that the anti-IL-2 antibody S4B6, previously thought to be neutralizing, actually enhances the potency of IL-2 *in vivo* for T cells bearing the intermediate affinity receptor for IL-2, IL2Rβγ (ref. 27). In that study, co-administration of small amounts of recombinant IL-2/anti-IL-2 immune complexes resulted in the delivery of a potent signal through IL2Rβγ that was independent of IL-2Rα. To determine whether IL-2/anti-IL-2 immune complexes could deliver functional IL-2 signals during acute infection, we injected 1×10^4 naive WT and IL-2Rα-deficient P14 cells into B6 mice as described previously (Supplementary Fig. 3), infected them with LCMV and treated mice with IL-2/anti-IL-2 immune complexes or with anti-IL-2 alone. WT and IL-2Rα-deficient P14 cells expanded to similar levels by day 8 post-infection in all treatment groups (data not shown). As seen previously (Fig. 2d), by day 19 post-infection, 35–40% of IL-2Rα-deficient cells expressed CD62L in untreated mice, compared to 10–13% of WT P14 cells. In contrast, IL-2Rα-deficient P14 cells in mice treated during the primary response with IL-2/anti-IL-2 immune complexes or with anti-IL-2 alone had levels of CD62L expression that were similar to WT P14 cells (Fig. 4a), indicating that the signalling of IL-2/anti-IL-2 immune complexes through IL2Rβγ can provide a functional IL-2 signal during acute infection.

Because the activity of the complexes is short-lived27, we were able to design experiments in which the timing of IL-2 signals to IL-2Rα-deficient T cells could be manipulated. WT/*IL-2Rα*$^{-/-}$ mixed chimaeras were infected with LCMV and either left untreated, injected with anti-IL-2 alone or injected with IL-2/anti-IL-2 complexes daily for the first six days of infection. Both WT and IL-2Rα-deficient CD8 T cells developed comparable effector and memory populations (data not shown). Six weeks after infection mice were re-challenged with a high dose of LM-GP, and further treatment groups received daily injections of IL-2/anti-IL-2 immune complexes or anti-IL-2 alone during the secondary response. Treatment with IL-2/anti-IL-2 immune complexes or with anti-IL-2 alone during the primary response rescued the ability of IL-2Rα-deficient CD8 memory cells to respond to re-challenge. Conversely, treatment with immune complexes during the secondary response only modestly improved the expansion of IL-2Rα-deficient CD8 memory cells, while treatment with anti-IL-2 alone had no effect (Fig. 4b,c). We conclude that IL-2 signalling during the primary response programmed the formation of fully responsive CD8 memory cells capable of generating robust recall responses.

To determine whether IL-2 signals to $CD8^+$ T cells were autocrine, we generated mixed chimaeras with a mix of B6 and IL-2-deficient bone marrow. In this scenario, IL-2-deficient T cells are dependent on paracrine IL-2 signalling. We observed no differences in either the primary or secondary responses of WT and IL-2-deficient T cells, demonstrating that paracrine IL-2 signalling is sufficient for the development of fully functional $CD8^+$ memory cells (Supplementary Fig. 8). What cell type, then, is the source of IL-2 for promoting the development of functional $CD8^+$ memory T cells? One candidate is the CD4 subset, because activated $CD4^+$ T cells are the major source of IL-2 *in vivo*. Alternatively, IL-2 may come from other sources, including other activated $CD8^+$ T cells, as well as activated dendritic cells28.

Signals delivered during the programming of immune responses can shape the long-term fate and function of CD8 memory cells15,16, and we show here that IL-2 signalling during the primary response to acute infection affects the ability of the subsequently arising $CD8^+$ memory T cell population to survive and accumulate following secondary antigen exposure. Future studies should focus on the specific pathways by which IL-2 signalling during the primary response promotes enhanced survival of $CD8^+$ T cells during the recall response. More experiments are also required to delineate the role of IL-2 in a broad spectrum of *in vivo* immune responses, including those directed towards other types of acute and chronic infections, tumours, and protein and DNA immunizations. Resolving these questions will be an important step in understanding the mechanisms by which fully functional $CD8^+$ memory T cells are generated.

Methods

Mice and infections

6–8-week-old C57BL/6 ($Thy1.2^+$), B6.SJL-*PtprcaPep3b*/BoyJ ($CD45.1^+$) and B6.PL-*Thy1a*/CyJ ($Thy1.1^+$) mice, and 4-week-old B6.129P2-*Il2tm1Hor*/J (IL-2-deficient) and B6.129S4-*Il2ratm1Dw*/J (IL-2Rα-deficient) mice were purchased from Jackson Laboratories. P14 T-cell-receptor transgenic mice were bred at our facilities. Mice were infected with LCMVArmstrong, LM-Ova or LM-GP (provided by H. Shen, Univ. Pennsylvania), as described17,18. Viral and bacterial stocks were prepared and propagated as described18,29. Mice were injected intraperitoneally daily with 50μg anti-IL-2 (clone S4B6) alone or anti-IL-2 and 1.5μg recombinant mouse IL-2 (eBiosciences) as described27.

Generation of mixed bone marrow chimaeras

Bone marrow preparations from femur and tibia were incubated with anti-CD3-biotin antibody and anti-biotin magnetic beads, followed by bead depletion on an AutoMacs (Miltenyi). 5×10^6 T-cell-depleted bone marrow cells from each of the indicated donors were injected intravenously into lethally irradiated B6 hosts (1,000 rad). Mice were infected 8–12 weeks post-transplant.

BrdU and CFSE labelling

Mice were fed 0.8 mg ml^{-1} BrdU (Sigma) in their drinking water for 7 days and stained for BrdU incorporation according to the manufacturer's instructions (BD Pharmingen). Splenocytes were labelled in 5μM CFSE (Molecular Probes) for 7 min in warm RPMI.

Cell preparations and *ex vivo* restimulations

For transfer of P14 cells, $CD44^{hi}$ cells were depleted by incubation with anti-CD44-biotin, followed by magnetic bead depletion with anti-biotin beads (Miltenyi). *Ex vivo* restimulations were performed as described30 and intracellular cytokine staining was carried out according to the manufacturer's instructions (Cytofix/Cytoperm kit, BD Pharmingen).

Antibody staining and flow cytometry

Cells were stained with directly conjugated antibodies purchased from BD Pharmingen or eBiosciences and analysed on either a FACSCalibur or FACSCanto flow cytometer (Becton Dickinson). Two-way cell sorting was performed on a FACSAria (Becton Dickinson).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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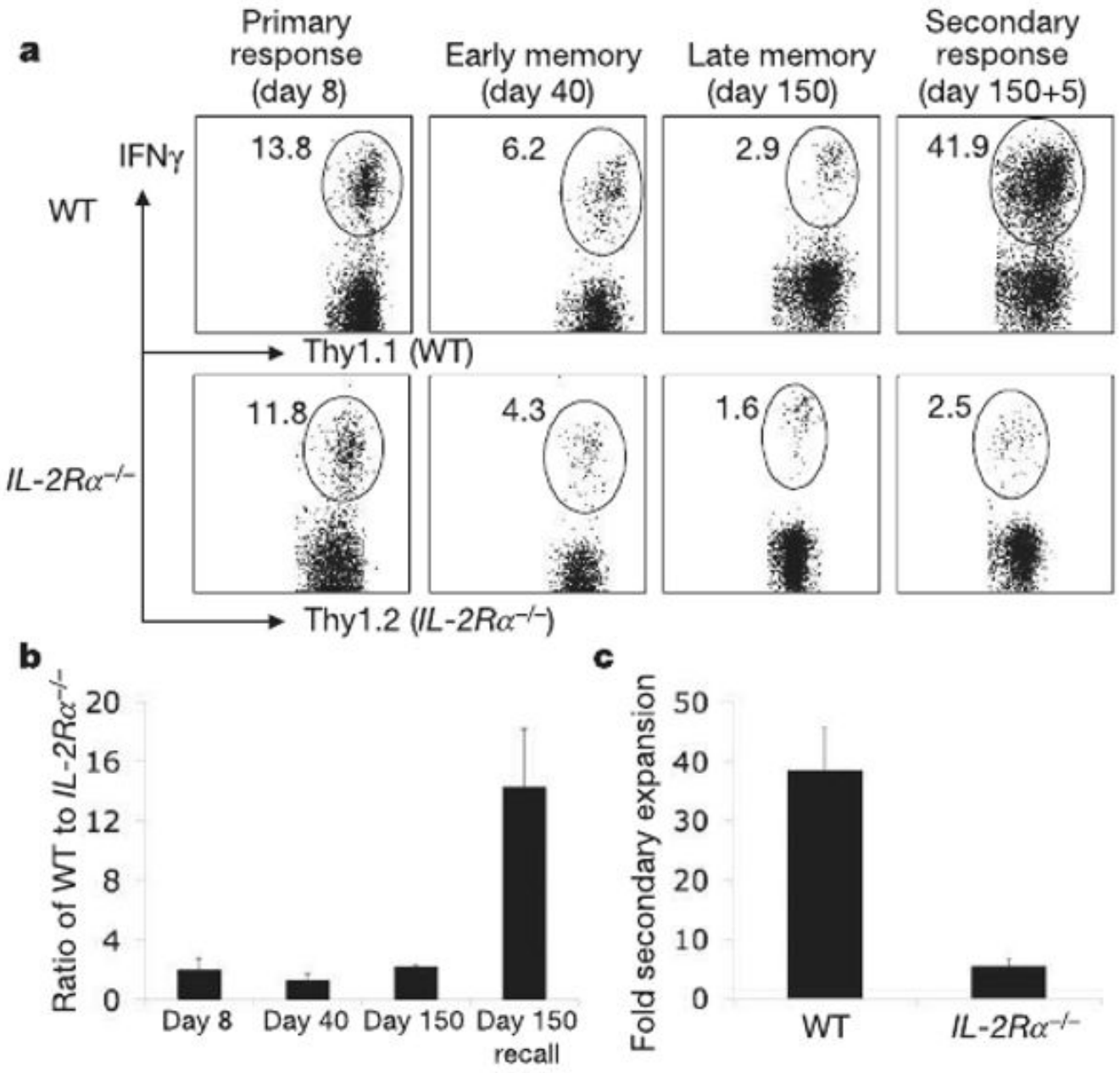

Figure 1. IL-2Rα-deficient CD8$^+$ T cells generate robust primary but defective secondary responses

a, WT/*IL-2Rα*$^{-/-}$ mixed chimaeras were infected with 2×10^5 plaque-forming units (PFU) LCMV and assessed for the frequency of interferon-γ (IFNγ)-producing cells specific for the $GP_{33–41}$ epitope among either WTor IL-2Rα-deficient CD8$^+$ T cells in the spleen. In all experiments, CD45.1$^+$ host cells were excluded. Mice were re-challenged 150 days post-infection with 1×10^5 colony-forming units (CFU) LM-GP, and $GP_{33–41}$-specific responses in the spleen were analysed 5 days later (day 150 + 5). **b**, The ratio of $GP_{33–41}$-specific WT to IL-2Rα-deficient responders is shown at each time point. **c**, The fold expansion by 5 days after re-challenge is shown for WT and IL-2Rα-deficient T cells. Error bars display s.e.m. (n = 3–4) and results are representative of five separate time courses.

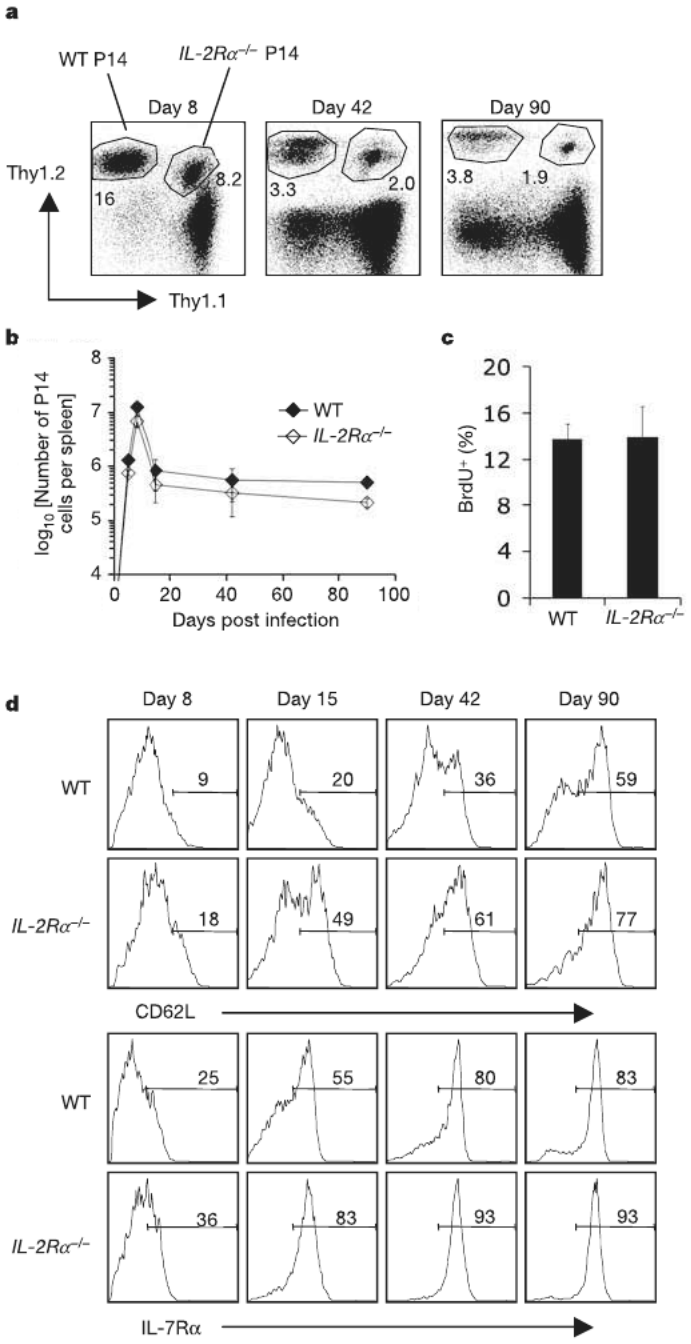

Figure 2. IL-2Rα-deficient memory cells are maintained at normal levels

a, 1×10^4 naive WT and IL-2Rα-deficient P14 cells were co-transferred into B6 hosts, infected with LCMV, and frequencies of P14 cells were measured in the spleen. **b**, The total number of WT or IL-2Rα-deficient P14 cells in the spleen is shown over a 90 day time course. **c**, At day 90 post-infection, mice were fed BrdU in their drinking water for 7 days and stained for BrdU incorporation by WT or IL-2Rα-deficient P14 cells. **d**, Cell surface expression of the indicated molecules by WT or IL-2Rα-deficient P14 cells at days 8, 15, 42 and 90 post-infection. Results are representative of 3 separate time courses and error bars display s.e.m. (n = 3–4).

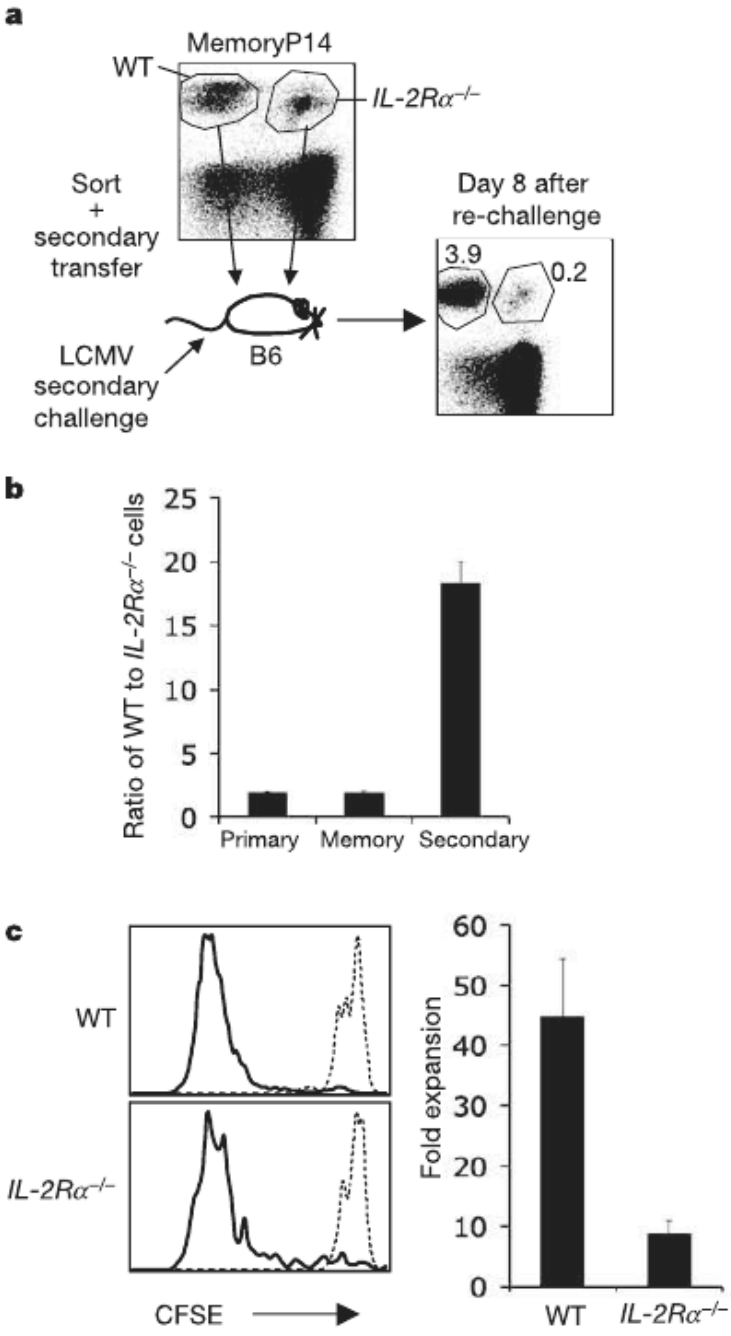

Figure 3. IL-2Rα-deficient memory cells proliferate but do not accumulate following rechallenge

a, At 42 days post-infection, WT and IL-2Rα-deficient memory P14 cells were sorted, transferred into new B6 hosts (1×10^4 of each) and rechallenged with LCMV. Eight days after secondary challenge, we measured their frequency in the spleen. Results were consistent for four separate experiments. **b**, The ratio of WT to IL-2Rα-deficient P14 cells was assessed at days 8 and 42 post-infection, as well as day 8 after re-challenge. Error bars display s.e.m. (*n* = 3–5) and are representative of four separate experiments. **c**, At day 90 post-infection, WT and IL-2Rα-deficient memory P14 cells were labelled with CFSE, transferred to a new host and re-challenged with LCMV. Flow plots indicate CFSE divisions after three days, with

dashed lines representing uninfected controls, and the graph displays the fold expansion of each group. Error bars display s.e.m. (n = 3).

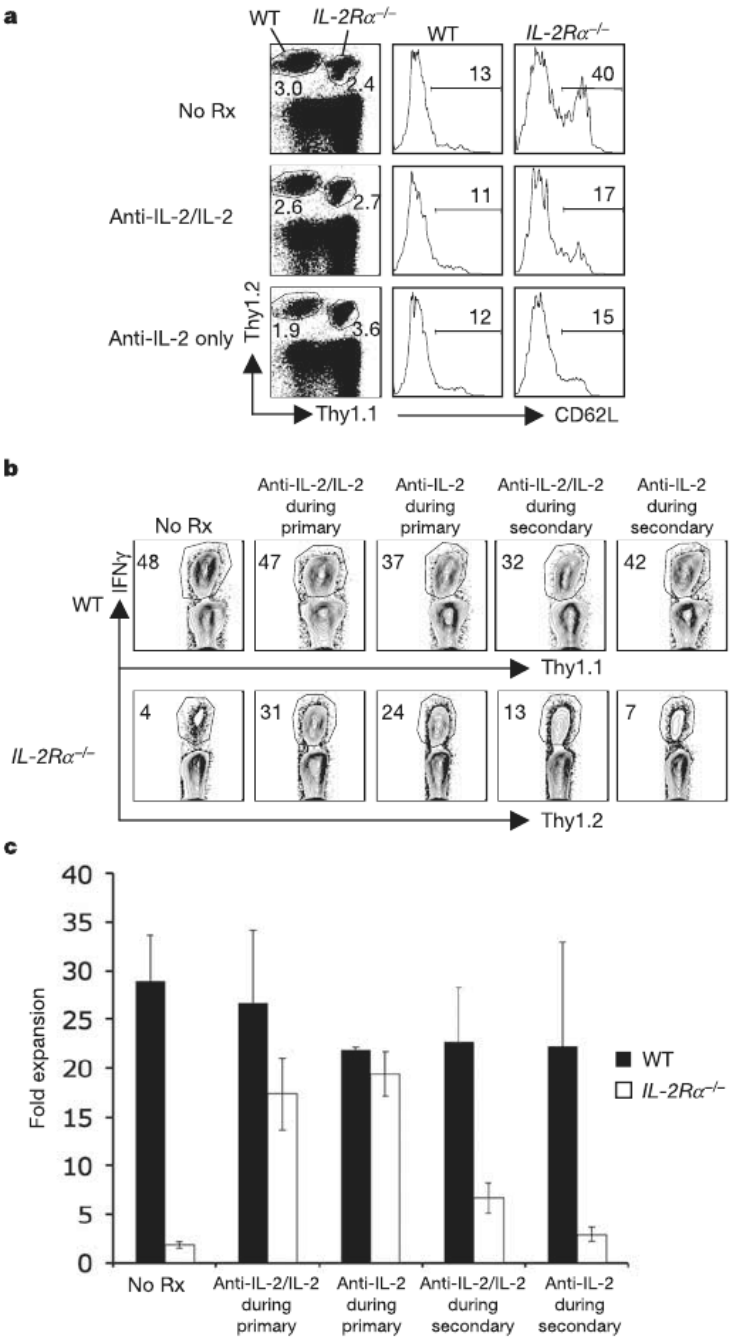

Figure 4. IL-2 signalling during the primary response promotes secondary CD8$^+$ T cell responsiveness

a, Following co-transfer of 1×10^4 naive WT and IL-2Rα-deficient P14 cells, B6 hosts were infected with LCMV and received daily intraperitoneal injections of 50μg anti-IL-2 (clone S4B6) alone, daily co-injections of anti-IL-2 and 1.5μg recombinant mouse IL-2 on days 0–6 of the primary infection, or no treatment (No Rx). Plots display the frequency and CD62L expression of WT and IL-2Rα-deficient P14 cells at day 19 post-infection. **b**, WT/*IL-2Rα*$^{-/-}$ mixed chimaeras were infected with LCMV and re-challenged with LM-GP 6 weeks later. Mice were treated on either days 0–6 of the primary infection or days 0–4 of the secondary infection. Plots display the frequency of $GP_{33–41}$-specific WT or IL-2Rα-deficient CD8$^+$ T

cells at day 5 post-rechallenge. **c**, The graph displays the fold expansion by 5 days post-rechallenge. Error bars display s.e.m. (n = 3).