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IL-2R α mediates temporal regulation of IL-2 signaling and enhances immunotherapy

Ee W. Su^{1,†}, Caitlin J. Moore^{1,†}, Samantha Suriano¹, Christopher B. Johnson¹, Neizel Songalia¹, Alicia Patterson¹, Daniel J. Neitzke¹, Kristina Andrijauskaite¹, Elizabeth Garrett-Mayer³, Shikhar Mehrotra¹, Chrystal M. Paulos², Andrew L. Doedens⁴, Ananda W. Goldrath⁴, Zihai Li², David J. Cole¹, and Mark P. Rubinstein^{1,2,*}

¹Department of Surgery, Medical University of South Carolina, Charleston, SC

²Department of Microbiology and Immunology, Medical Univ. of South Carolina, Charleston, SC

³Department of Public Health Sciences, Medical Univ. of South Carolina, Charleston, SC

⁴Division of Biological Sciences, University of California, San Diego

Abstract

IL-2 is a lymphocyte growth factor that is an important component of many immune-based cancer therapies. The efficacy of IL-2 is thought to be limited by the expansion of T regulatory cells, which express the high affinity IL-2 receptor subunit, IL-2R α . IL-15 is under investigation as an alternative to IL-2. Although both cytokines signal through IL-2R $\beta\gamma$, IL-15 does not bind IL-2R α and therefore induces less T regulatory cell expansion. However, we found that transferred effector CD8⁺ T cells induced curative responses in lymphoreplete mice only with IL-2-based therapy. While conventional *in vitro* assays showed similar effector T cell responsiveness to IL-2 and IL-15, upon removal of free cytokine, IL-2 mediated sustained signaling dependent on IL-2R α . Mechanistically, IL-2R α sustained signaling by promoting a cell-surface IL-2 reservoir and recycling of IL-2 back to the cell surface. Our results demonstrate that IL-2R α endows T cells with the ability to compete temporally for limited IL-2 via mechanisms beyond ligand affinity. These results suggest that strategies to enhance IL-2R α expression on tumor-reactive lymphocytes may facilitate the development of more effective IL-2-based therapies.

*Correspondence: Mark P. Rubinstein, PhD, Assistant Professor, Surgical Oncology, Department of Surgery, Medical University of South Carolina, 86 Jonathan Lucas St, HO506, Charleston, SC 29425, Tel: 843-792-1451; Fax: 843-792-2556, markrubinstein@musc.edu.

[†]These authors contributed equally to this work.

Author contributions.

EWS and CJM designed research, performed experiments, analyzed data, and wrote the manuscript. SS, CBJ, NS, AP, DJN, and KA designed research, performed experiments, analyzed data, and edited the manuscript. EGM designed experiments, analyzed data, performed statistical analysis, and edited the manuscript. SM, CMP, ALD, AWG, ZL, and DJC designed experiments, analyzed data and edited the manuscript. MPR designed experiments, performed experiments, analyzed data, wrote the manuscript, and supervised the study.

Competing interests.

DJC and MPR are co-inventors on a provisional patent application related to genetically modifying lymphocytes with IL-2 receptor genes. The other authors declare that they have no competing interests.

Introduction

The administration of IL-2 is an important component of many cancer immune therapy strategies including adoptive T cell transfer (1–4). Despite its widespread use, the efficacy of IL-2 is limited by short half-life, toxicity, and expansion of IL-2R α^{hi} T regulatory cells. IL-15 is a promising alternative. Like IL-2, IL-15 signals exclusively through the intermediate affinity IL-2R $\beta\gamma$ subunits (CD122/CD132). However, for high affinity cytokine binding, IL-2 and IL-15 utilize specific IL-2R α (CD25) and IL-15R α subunits. This differential α -chain dependence likely dictates the distinct biological outcomes associated with IL-2 and IL-15 (5, 6). In the case of the latter, membrane-bound IL-15R α can lead to the recycling of IL-15, which sustains cellular signaling and lymphocyte survival (7). However, despite homology with IL-15R α (8), IL-2R α is not thought to facilitate sustained signaling or cytokine recycling due to lower affinity for IL-2 (2–4). While briefly expressed on activated lymphocytes, IL-2R α is constitutively highly expressed on T regulatory cells. For this reason, IL-2 but not IL-15 is essential for T regulatory cell survival and expansion, and mice deficient in IL-2 or IL-2R α develop T cell-mediated autoimmunity (9, 10). In contrast, mice deficient in IL-15 or IL-15R α are relatively healthy with reduced frequencies of CD8 $^{+}$ memory-phenotype cells and NK cells (11, 12). Therefore, given the potential undesirable consequences of engaging the IL-2R α pathway, we hypothesized that IL-15-based therapy would most efficiently augment the efficacy of adoptively transferred tumor-reactive effector CD8 $^{+}$ T cells, particularly in lymphoreplete mice with an intact T regulatory cell population.

Results

IL-2- but not IL-15- therapy mediates anti-tumor immunity after adoptive transfer of activated CD8 $^{+}$ T cells

To assess the impact of cytokine therapy on adoptively transferred effector CD8 $^{+}$ T cells, we used IL-2/anti-IL-2 mAb (IL-2/mAb) and IL-15/sIL-15R α -Fc (IL-15/sIL-15R α) complexes, in which the antibody or receptor acts as a carrier molecule to improve the half-life and biological activity of free cytokine *in vivo* (13–15). To test effector T cell responsiveness to cytokines in a clinically relevant model, B6 mice were injected (s.c.) with B16 melanoma tumor cells (Fig. 1a). After the establishment of palpable tumors, unirradiated mice received activated IL-12-conditioned T cells (Tc1) from pmel-1 TCR transgenic mice, from which CD8 $^{+}$ T cells recognize an endogenous B16 tumor antigen (H-2D $^{\text{b}}$ -restricted gp100 $_{25-33}$ peptide). We have shown these Tc1 effector cells are highly efficacious against tumor in lymphodepleted mice (16). For the first week after adoptive transfer, IL-15/sIL-15R α or IL-2/mAb (clone 5355) complexes were administered every 48 hours. While 6 of 9 mice that received IL-2/mAb complexes were cured of established tumor, mice that received either IL-15/sIL-15R α complexes or no cytokine therapy showed no tumor regression (Fig. 1b). To better understand this differential response, we assessed the persistence of donor Tc1 cells in recipients that received treatment with IL-2/mAb complexes or IL-15/sIL-15R α complexes. Independent of the presence of tumor, only IL-2/mAb complexes enhanced the persistence of effector CD8 $^{+}$ T cells in a systemic fashion across multiple organs (Fig. 1c and Supplementary Fig. 1a). Notably, without lymphodepletion or vaccination, we routinely

achieved sustained donor T cell frequencies of 20% or higher in the peripheral blood. Furthermore, donor Tc1 cells were equally functional across treatment groups as indicated by the ability to produce $\text{IFN}\gamma$ and $\text{TNF}\alpha$ (Supplementary Fig. 1b). Finally, as a control, we found that the transfer of tumor-reactive effector CD8^+ T cells was necessary for curative therapy. Thus, tumor-bearing mice treated with only IL-2/mAb or IL-15/sIL-15R α complexes exhibited minimally delayed tumor growth, albeit comparable between cytokine conditions (Supplementary Fig. 2).

Donor T cell expression of IL-2R α is critical for preferential IL-2-mediated responses

The preferential response of effector CD8^+ T cells to IL-2/mAb but not IL-15/sIL-15R α complexes was contrary to our expectation. This response was not dose related as IL-2/mAb and IL-15/sIL-15R α complexes expanded IL-2R $\beta\gamma^{\text{hi}}$ cells such as memory-phenotype CD8^+ T cells and NK cells to a similar extent *in vivo* (Supplementary Fig. 3) (17). However, only IL-2/mAb complexes expanded T regulatory cells (Supplementary Fig. 3), which are characterized by their expression of IL-2R α . As IL-12-conditioned (Tc1) effector CD8^+ T cells express very high levels of IL-2R α (16), our results suggested an unappreciated role for cell surface IL-2R α on effector T cells in dictating responsiveness to IL-2 therapy. To formally test this, we made use of two anti-IL-2 mAbs with the ability to differentially redirect IL-2 based on lymphocyte cell surface IL-2R α expression. IL-2/mAb_{CD25} complexes (clone 1A12) preferentially expand IL-2R α^{hi} lymphocytes, while IL-2/mAb_{CD122} complexes (clone S4B6) act in an IL-2R α -independent manner (15, 18). We tested these two complexes in lymphoreplete mice injected with Tc1 cells. For only this experiment, we generated Tc1 cells from another TCR transgenic mouse, OT-I, to confirm our results with a different TCR. While IL-2/mAb_{CD122} complexes mediated a minimal increase in persistence, IL-2/mAb_{CD25} complexes induced donor T cell levels of greater than 60% of total lymphocytes (Fig. 1d). To further confirm that this effect was dependent on IL-2R α and not on IL-12 conditioning or selective TCR engagement, we stimulated polyclonal T cells from wildtype mice with plate-bound anti-CD3 mAb, a method that generates IL-2R α^{hi} effector CD8^+ T cells. Upon adoptive transfer into lymphoreplete mice, IL-2/mAb complexes (clone 5355) greatly enhanced the persistence of polyclonal T cells (Fig. 1e). Finally, as an additional control, Tc0 cells, which have lower levels of surface IL-2R α (16), showed limited IL-2/mAb-driven persistence (Supplementary Fig. 4).

IL-2R α induces sustained IL-2 signaling in effector CD8^+ T cells after cytokine withdrawal

To uncover the mechanism behind the remarkable IL-2R α -dependent responsiveness of effector Tc1 cells *in vivo*, we assayed IL-2 and IL-15 activity downstream of IL-2R $\beta\gamma$ using standard *in vitro* assays quantifying phosphorylation of STAT5 (a proximal signaling event), viability, and proliferation (Fig. 2a). In the context of STAT5 phosphorylation in response to titrated cytokine, we found that Tc1 (IL-2R α^{hi}) cells exhibited marginally increased sensitivity to IL-2 versus IL-15 when compared to Tc0 effector cells (IL-2R α^{med}) (Fig. 2b,c), which is consistent with previous findings (19). The addition of a blocking antibody (anti-IL-2R α mAb, PC61 clone) also showed a minimal benefit of IL-2R α engagement on Tc1 cells in comparison between titrated IL-2 and IL-15 (Supplementary Fig. 5). Notably, Tc1 cells responded comparably to IL-2 and IL-15 in standard assays of proliferation and viability (Supplementary Fig. 6). Importantly, there was no difference in the kinetics of

STAT5 phosphorylation between cells cultured in IL-2 or IL-15 (Fig. 2d). The mildly enhanced sensitivity of Tc1 cells to IL-2 versus IL-15 seemed unlikely to account for the dramatic difference in activity observed *in vivo*. Therefore, we hypothesized that IL-2R α does not simply improve cellular affinity for IL-2, but allows for sustained IL-2 signaling after a T cell transitions from a cytokine-rich to a cytokine-free environment. To test this idea, we used a cytokine pulse assay. Tc1 and Tc0 cells were cultured overnight with a saturating dose of IL-2 or IL-15, washed, and replated without cytokine as shown in Figure 2e. Consistent with our hypothesis, only pre-culture of Tc1 cells with IL-2 led to sustained STAT5 phosphorylation in the absence of additional cytokine (Supplementary Fig. 7). To directly test the role of IL-2R α in promoting sustained signaling on effector CD8⁺ T cells, we cultured Tc1 cells for 90 minutes with IL-2 in the absence or presence of blocking anti-IL-2R α antibody (PC61 clone). This shorter pulse was equally sufficient for inducing sustained signaling as indicated by STAT5 phosphorylation (Fig. 2f). Importantly, blockade of IL-2R α completely abolished the sustained IL-2 signaling as indicated by STAT5 phosphorylation and proliferation (Fig. 2f and Supplementary Fig. 8 & 9). Polyclonal effector CD8⁺ T cells activated in the absence of IL-12 also showed sustained IL-2 signaling, and importantly, effector cells generated from IL-2R α ^{+/-} mice showed roughly half the sustained IL-2 signaling (Fig. 2g). To ensure that these cells had similar IL-2R $\beta\gamma$ signaling potential, we pulsed wildtype and IL-2R α ^{+/-} effector CD8⁺ T cells with IL-15 and found no differences in their response (Fig. 2h). Notably, the ability to induce sustained IL-2 signaling on mouse effector cells was observed with human and mouse IL-2 (Supplementary Fig. 10). Furthermore, culture of human effector T cells with hIL-2 but not hIL-15 led IL-2R α -dependent sustained STAT5 phosphorylation (Supplementary Fig. 11). Finally, to verify that IL-2/mAb complexes (clone 5355) used in our *in vivo* experiments were permissive to engagement of IL-2R α , we repeated the pulse assay with hIL-2 and excess anti-IL-2 mAb. *In vitro* generated IL-2/mAb complexes induced sustained IL-2 signaling that was dependent on IL-2R α (Supplementary Fig. 12a). In contrast, IL-2/mAb_{CD122} complexes (clone S4B6), which do not engage IL-2R α (15, 18), failed to induce sustained signaling *in vitro* (Supplementary Fig. 12b).

IL-2R α facilitates sustained IL-2 signaling through creation of an extracellular reservoir and recycling

To understand how IL-2R α promotes sustained IL-2 signaling, we hypothesized two non-mutually exclusive possibilities. First, IL-2R α may bind IL-2 and create a cell-surface cytokine reservoir due to the high ratio of surface IL-2R α to IL-2R $\beta\gamma$, as IL-2/IL-2R α internalization can only occur in the presence of both IL-2R β and γ (20, 21). Such a reservoir of IL-2 bound to IL-2R α would mediate gradual signaling by continually feeding the rate-limiting, endocytosed IL-2R $\beta\gamma$. In support of this possibility, we detected high surface levels of IL-2 on effector CD8⁺ T cells that gradually waned after extended culture, and this cell-surface IL-2 was dependent on available IL-2R α (Fig. 3a,b). Furthermore, antibodies against IL-2 added after the removal of free cytokine from IL-2 pulsed cells were able to dampen sustained signaling (Fig. 3c). A second possible way in which IL-2R α might sustain signaling is by promoting recycling of IL-2 from within the cell to the surface, thus allowing for repetitive signaling. To test this hypothesis, Tc1 cells were pulsed with IL-2 at 37°C to allow for cytokine internalization. Cells were then stripped of surface IL-2 using an

acid wash. Upon reculture at 37°C, we were able to detect re-appearance of either mIL-2 or hIL-2 on the cell surface (Fig. 3d). Minimal surface IL-2 was observed when cells were pulsed at 4°C or on the surface of mixed bystander Tc1 cells (Fig. 3e). Importantly, the species-specificity of our reagents precluded autocrine production as the source of cell surface IL-2 after acid wash (Supplementary Fig. 13). In additional support of IL-2R α -mediated recycling, we observed sustained pSTAT5 signaling after acid washing of cells pulsed with hIL-2 at 37°C but not 4°C (Fig. 3f). Because internalization of IL-2R $\alpha\beta\gamma$ does not occur at 4°C, these data provide further support that sustained signaling occurs in part through an IL-2R α bound pool of internalized IL-2. It is notable that we could not block sustained STAT5 signaling in cells pulsed with mIL-2 at 4°C followed by acid washing, possibly reflecting a higher affinity of mIL-2 for mIL-2R α compared with that of hIL-2 for mIL-2R α (18, 22). Finally, confocal microscopy showed discrete punctate structures of either mIL-2 or hIL-2 when cells were incubated with cytokine at 37°C but not 4°C (Supplementary Fig. 14 & 15). These punctate structures colocalized with IL-2R α , Rab5, and EEA1, but less frequently with LAMP-1, consistent with intracellular IL-2 being accessible to the recycling pathway (Fig. 3g,h and Supplementary Fig. 16) (23, 24). Taken together, these results suggest that IL-2R α both promotes an extracellular reservoir for IL-2 and mediates recycling of IL-2.

IL-2R α expression on donor CD8⁺ T cells provides a competitive advantage to IL-2 therapy in a lymphoreplete but not lymphopenic host environment

Our results thus far suggest that the differential responsiveness of Tc1 cells to IL-2- and IL-15 therapy *in vivo* is a consequence of IL-2R α on donor T cells providing a competitive advantage to accessing cytokine. To formally test this hypothesis, we initially attempted to activate T cells from wildtype and IL-2R $\alpha^{-/-}$ mice. However, this proved technically not feasible for us as T cells isolated from IL-2R $\alpha^{-/-}$ mice were resistant to normal activation, likely due to the immune alterations in the absence of IL-2 responsiveness (9). Therefore, we used polyclonal IL-2R $\alpha^{+/-}$ T cells, as these cells activated comparably to wildtype T cells and had approximately half the expression of IL-2R α (Fig. 4a). Using the Thy1.1 congenic marker to distinguish between genotypes, these two cell populations were mixed and adoptively transferred into non-irradiated B6(CD45.1) recipient mice. Mice were treated with IL-2/mAb or IL-15/sIL-15R α for 1 week. We hypothesized that IL-2R $\alpha^{+/-}$ donor CD8⁺ T cells would not persist as well as their wildtype counterparts due to loss of one allele. In contrast to our expectations, wildtype and IL-2R $\alpha^{+/-}$ donor T cells did not show differential responsiveness to treatment with IL-2/mAb or IL-15/sIL-15R α complexes (Fig. 4a, b). These results suggest a threshold of IL-2R α *in vivo*, both in terms of level and durability of expression, that when reached is sufficient for providing donor cells a competitive advantage to IL-2 therapy in a lymphoreplete environment.

As an alternative means of assessing the role of IL-2R α on donor T cells *in vivo*, we compared the responsiveness of IL-2R α^{hi} donor T cells to IL-2- and IL-15 therapy with the addition of lymphodepletion to destroy host cells. We predicted that the advantage of IL-2R α -competent cytokine therapy would be lost in the absence of host IL-2R $\beta\gamma^+$ lymphocytes competing for cytokine (Supplementary Fig. 17a). Thus, mice were given total body irradiation (600rad) prior to adoptive transfer of effector Tc1 CD8⁺ T cells, and then

treated for one week with IL-2/mAb and IL-15/sIL-15R α complexes. Consistent with our prediction, both IL-2 and IL-15 therapy effectively augmented the persistence of donor cells both in the blood and in the spleen, and only in lymphodepleted mice (Fig. 4c and Supplementary Fig. 17b). These results demonstrate a critical role for IL-2R α on donor T cells in promoting IL-2 responsiveness in a lymphoreplete host environment.

Discussion

Administration of IL-2 is a critical component of many T cell-based strategies for cancer therapy. However, IL-2 has a short half-life and toxicity at therapeutic doses. Furthermore, as IL-2 selectively expands T regulatory cells, it has been proposed that IL-15-based therapies may more effectively support adoptively transferred effector T cells. However, our results show that in the absence of lymphodepletion, only IL-2R α -competent cytokine therapy led to sustained persistence and anti-tumor immunity of effector CD8⁺ T cells expressing elevated IL-2R α . Interestingly, host cells appear to play an important role in this process, as in mice preconditioned with total body irradiation, IL-2R α -independent (or IL-15-based) cytokine therapy effectively augmented donor T cell responses. Together, these results suggest that IL-2R α expression on donor T cells allows them to outcompete host cells for limited amounts of IL-2. Our results have important implications for the utilization of cytokine-based therapeutics in human patients as efficacy of IL-2- and IL-15-based therapies will be dictated by donor cell cytokine receptor subunit expression and competition with host lymphocytes.

In addition to our clinically relevant findings, we have uncovered molecular mechanisms which explain how IL-2R α increases the durability of IL-2 signaling. It has been widely assumed that IL-2R α improves IL-2 responsiveness primarily by acting as a high affinity receptor. We find, however, that beyond ligand affinity, IL-2R α is able to promote sustained IL-2 signaling after withdrawal of cytokine, thereby providing a temporal dimension to IL-2 signaling. To enable sustained signaling, we find at least two distinct IL-2R α -dependent mechanisms, including the creation of a cell-surface cytokine reservoir and recycling of IL-2 back to the cell surface. Because it is known that the IL-2R α subunit is recycled after cytokine-mediated internalization, instead of being degraded like the IL-2R $\beta\gamma$ subunits (25), our results suggest a mechanism by which IL-2R α can rescue IL-2 from degradation. These properties may be relevant during early stages of infection when lymphocytes express high levels of IL-2R α (26–28).

Our results with IL-2R α are reminiscent of the ability of membrane-bound IL-15R α to facilitate recycling and sustained cellular responsiveness to IL-15 (7). Interestingly, mutants of IL-2, selected for high affinity binding to IL-2R α (and thus thought to more resemble IL-15 in terms of high affinity for cognate alpha receptor), do exhibit prolonged cytokine responsiveness when assayed on cell lines and this has been attributed to the creation of both an extracellular reservoir and recycling (29–31). Importantly, our findings are distinct as they show that wildtype IL-2 can induce sustained cytokine signaling on primary mouse and human lymphocytes. Furthermore, we find sustained IL-2-signaling is directly dependent on IL-2R α as shown by antibody-mediated blockade and genetic targeting.

There are emerging questions from our study that warrant additional investigation. Foremost, our results suggest strategies to enhance IL-2R α expression on donor T cells may allow for more effective IL-2-based anti-tumor responses. In this context, it will be important to assess the therapeutic potential of strategies that redirect IL-2 toward IL-2R α , approaches that improve the half-life of IL-2, and also the administration of low-dose IL-2. There also remain important biological questions from our mechanistic findings. For example, how does the presence of IL-2R α impact the fate of internalized IL-2 through different endocytic compartments? Our ability to image and track IL-2 by confocal microscopy may provide evidence in support of IL-2R α -mediated endosomal IL-2 recycling. Another question is related to IL-2-responsive T regulatory cells. Do cytokine-stimulated effector T cells have resistance to suppression by such T regulatory cells? Also important is whether IL-2R α can mediate sustained signaling on T regulatory cells or other cell types. This may depend on the ratio and localization of the different receptor subunits. It may also be relevant that ADP ribosylation can regulate IL-2 binding to IL-2R α (32).

In conclusion, our findings have important implications for efforts to boost the therapeutic value of IL-2- and IL-15-mediated signaling in patients with cancer as well as infectious disease. Our results suggest that in non-lymphopenic patients, IL-2R α -competent cytokine therapy will be effective if responding T cells express elevated levels of IL-2R α . In contrast, in lymphodepleted patients where host cells are destroyed and do not compete for cytokine availability, IL-2R α -independent signaling will likely be sufficient, and thus either IL-15 or IL-2-based therapies will mediate effective responses.

Materials and Methods

Study design

This was a preclinical study to assess the efficacy of cytokine therapy to augment anti-tumor T cell immune responses. We found that IL-2-based therapies were more efficacious than IL-15-based therapies in our tumor model, and thereafter, focused on understanding the mechanism of this differential response. For *in vivo* experiments, the numbers of mice are outlined in the figure legends. For all experiments, the number of independent replicates is outlined in each figure legend. Randomization and blinding for tumor experiments was done as described in the tumor methods below. Additional study design details are also included in the statistical analysis section below.

Recombinant proteins and antibodies

Human (h) IL-15, hIL-2, and anti-hCD3 mAb (clone OKT3) were kindly provided by the NCI Biological Resources Branch Preclinical Repository. Mouse (m) IL-2, mIL-12, and mIL-15 were purchased from Shenandoah Biotechnology. Recombinant sIL-15Ra-Fc (551-MR-100), anti-hIL-2 mAb (clone 5355), and anti-hIL-2 mAb (clone 5334) were purchased from R&D systems. Anti-mIL-2 mAb (clone 5B9-2-1) was purchased from Peprotech. Anti-hIL-2 mAb_{CD25} (clone 5344.111) was obtained from BD Bioscience. Anti-mIL-2 mAb_{CD122} (clone S4B6) and anti-IL-2R α (clone PC61) were obtained from Bioxcell. Anti-mIL-2 mAb_{CD25} (clone JES6-1A12), anti-mCD3 mAb (clone 145-2C11), and anti-mCD28

mAb (clone 37.51) were obtained from the UCSF monoclonal antibody core. Antibodies used for flow cytometric and confocal analysis are described below.

Mice and tumor cells

C57BL/6 (B6), B6.PL (Thy1.1), B6(CD45.1), pmel-1 TCR transgenic, and OT-I TCR transgenic mice were purchased from Jackson Laboratory. All animals were housed under specific pathogen-free conditions in accordance with institutional and federal guidelines. For tumor experiments, B16-F1 cells were obtained from ATCC.

T cell cultures

Mouse Tc1 and Tc0 cells were generated from pmel-1 and OT-I TCR transgenic mice as previously described (16). Briefly, splenocytes were cultured for three days with relevant peptide (for pmel-1, hgp100₂₅₋₃₃ peptide (KVPRNQDWL) and for OT-I, OVA₂₅₇₋₂₆₄ peptide (SIINFEKL)) and cultured with (Tc1) or without (Tc0) mIL-12 (10ng/ml). Polyclonal mouse T cells were generated by culturing B6 splenocytes for three days with plate-bound anti-CD3 mAb (clone 145-2C11, 1ug/ml) unless otherwise stated. Activated human T cells were generated by culturing de-identified human PBMCs (Research Blood Components) from healthy adult donors for two or three days with plate-bound anti-CD3 mAb (clone OKT3, 1ug/ml).

Tumor and persistence studies in mice

For tumor experiments, B6 mice were challenged subcutaneously with 2.5×10^5 B16-F1 tumor cells. Prior to randomizing mice to treatment groups, some mice were excluded due to abnormal tumor growth. As indicated, mice were treated by adoptive transfer of activated T cells (Tc1 or Tc0) by intravenous tail vein injection. Cytokine complexes were administered by intraperitoneal injection on days 0, 2, 4, and 6 after adoptive transfer unless otherwise indicated. Cytokine complexes used include: hIL-15/sIL-15R α , hIL-15 (0.5ug)/sIL-15R α -Fc (2.3ug); hIL-2/mAb, hIL-2 (1.5ug)/anti-IL-2 mAb (7.5ug, clone 5355); hIL-2/mAb_{CD25}, hIL-2 (1.5ug)/anti-IL-2 mAb (7.5ug, clone 5344.111); mIL-2/mAb_{CD122}, mIL-2 (1.5ug)/anti-IL-2 mAb (7.5ug, clone S4B6); and mIL-2/mAb_{CD25}, mIL-2 (1.5ug)/anti-IL-2 mAb (7.5ug, clone JES6-1A12). Tumor growth was measured by caliper every 2–4 days by personnel blinded to the treatment regimen. Tumor surface area (mm²) was calculated as length x width. Mice were sacrificed when tumors reached 400mm². For persistence studies, mice received adoptive transfer of activated T cells (Tc1 or Tc0). Peripheral blood lymphocytes or indicated organs were stained for CD8 and either Thy1.1 or CD45.1 to identify donor T cells. In experiments with a mixed transfer, we used effector T cells from wildtype (Thy1.1) and IL-2R α ^{+/-} (Thy1.2) mice that were activated with plate-bound anti-CD3/anti-CD28 mAb, mixed, and transferred into B6(CD45.1) mice. Where indicated, mice also received total body irradiation (600rad) one day prior to adoptive T cell transfer. In all adoptive transfer experiments, donor and recipient mice were gender-matched and recipient mice were 6 to 12 weeks of age. All animals were housed under specific pathogen-free conditions in accordance with institutional and federal guidelines.

Flow cytometry

Flow cytometry analysis was performed as previously described (17). The antibodies used in this study include CD8 (53–6.7), CD25 (PC61), CD45.1 (A20), IFN γ (XMG1.2), STAT5 pY694 (47/Stat5(pY694)), Thy1.1 (A20), and TNF α (TN3-19.12). These were purchased from BD Bioscience, Biolegend (San Diego, CA), and eBioscience (San Diego, CA). For analysis of phosphorylation of STAT5, we followed the manufacturer's protocol using Lyse/Fix and PermIII buffer (BD Bioscience). To examine cellular proliferation, cells were fixed and permeabilized according to the manufacturer's protocol for Cytotfix/Cytoperm (BD Bioscience) and stained with anti-Ki67 mAb (SolA15, eBioscience). Alternatively, BrdU (10 μ m) was added one hour prior to harvest, and cells were analyzed for BrdU incorporation as previously described (17). For Foxp3 staining, we followed the protocol outlined in the Foxp3 kit (eBioscience). Flow cytometry was performed on BD LSRII and BD FACSAccuri. Data were analyzed using FlowJo software (TreeStar). In all experiments, initial gating of live cells was performed using forward scatter and side scatter parameters, and cells were then gated on live lymphocytes. Isotype and fluorescence minus one (FMO) controls were performed as required. For experiments assessing IL-2, we always included control conditions without IL-2 pulsing.

In vitro experiments

For functional assays, Tc1 or Tc0 cells were incubated with cytokines and assayed for pSTAT5, Ki67, BrdU incorporation, or propidium iodide exclusion. For pulse assays, cells were incubated with or without cytokine at 200ng/ml at either 4°C or 37°C for 90 minutes unless otherwise indicated. Cells were then washed at least three times, replated without cytokine, and assayed for pSTAT5. When added during the pulse step, anti-IL-2R α mAb was added 15 minutes prior to cytokine addition. Acid wash was performed by washing cells twice for 2 minutes at 4°C with an acid wash buffer consisting of complete media adjusted to pH3.5 or pH3.75 with 1N HCl. For analysis of recycling of IL-2 to the cell surface, acid washed cells were replated in media at 37°C for the indicated amount of time with anti-IL-2 mAb conjugated to Alexa647 (5334 or 5B9-2-1 clone). To assess IFN γ and TNF α production, we added hgp100_{25–33} (1 μ g/ml) or PMA (50ng/mL) and ionomycin (1 μ M) to splenocytes for 5 hours in the presence of monensin (GolgiStop, BD Bioscience).

Confocal Microscopy

Tc1 cells were incubated with hIL-2 (200ng/ml), mL-2 (200ng/ml), or no cytokine, for 1 hour at either 4°C or 37°C unless otherwise stated. Cells were washed, fixed, and permeabilized using the Cytotfix/Cytoperm protocol. To determine the subcellular localization of internalized IL-2 by confocal microscopy, cells were stained with anti-hIL-2 mAb and either anti-IL-2R α pAb (R&D systems), anti-Rab5 mAb (C8B1, Cell Signaling), anti-LAMP1 mAb (1D4B, Biolegend), or anti-EEA1 mAb (C45B10, Cell Signaling). To detect anti-IL-2R α , we used an anti-goat IgG conjugated to Alexa488 (R&D systems). To detect EEA-1 and Rab5, we used an anti-rabbit IgG conjugated to Alexa488 (F(ab')₂ fragment, Cell Signaling). After washing, cells were transferred to SuperFrost microscope slides via cytospin. Immunofluorescence staining was visualized with a confocal microscope (Olympus Fluoview FV10i laser scanning confocal microscope system, Olympus) using a

60x water immersion objective (1.2 NA). Image analysis was performed using the FV10-ASW 1.7 software. In all images, IL-2 staining is presented as a red pseudocolor. In all experiments, cells pulsed without IL-2 were used as the primary control.

Statistical Analyses

Before analysis, graphical displays were made of all data vs. conditions to identify the need for transformations to adhere to model assumptions. For experiments comparing outcomes at a fixed point in time, log transforms were taken and comparisons of means performed using two-sample t-tests or linear regression (depending on the number of conditions). Where appropriate, t-tests assumed unequal variance across conditions. Comparisons of conditions where mice were followed over time were made at individual timepoints based on random effects linear regression models (with random effects to account for correlation of data from the same mouse over repeated measures) with the outcome (e.g. % T-cells) log-transformed. Graphical displays were used to assess appropriateness of transformation. Residual plots were inspected to assess assumptions of linear regression models. Time to sacrifice was compared across groups using log-rank tests. Time to sacrifice was compared across groups using log-rank tests. Percent colocalization was compared with log(percent) as the outcome (due to skewness) and main effects of LAMP-1 (vs. EEA-1) and rater. The LAMP-1 effect was evaluated based on the Wald test of the regression coefficient. Model results were exponentiated to provide point estimates for LAMP-1 and EEA-1 colocalization. In the interest of addressing our hypotheses and not over-testing, we did not perform hypothesis tests for every possible comparison in each figure. Instead, we focused on addressing our hypotheses and where comparisons were insignificant ($p > 0.05$) it is stated in the text; where tests were significant, it is stated and/or indicated with asterisks in figures. P-values are reported to two significant digits, except when the p-value is less than 0.001; for p-values smaller than 0.001, it is reported as ' $p < 0.001$ '. P-values are not corrected for multiple comparisons. For all analyses, statistical significance was based on a two-sided alpha level of 0.05. Statistical analyses were performed using Stata/IC (version 12.1) and R statistical software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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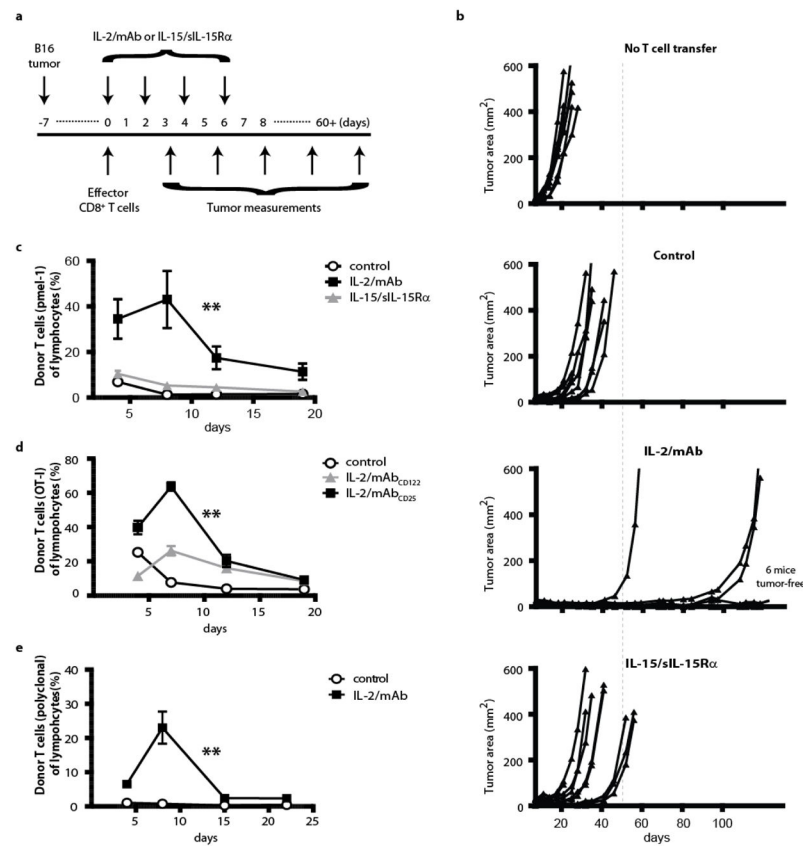


Figure 1. IL-2/mAb but not IL-15/sIL-15R α complexes induce potent effector T cell responses in tumor-bearing mice

(a) Treatment scheme for B6 mice injected s.c. with B16 melanoma tumor cells 7 days prior to the adoptive transfer of 3×10^6 pmel-1 Tc1 cells. Mice were then treated with hIL-2/mAb (clone 5355) or hIL-15/sIL-15R α complexes. (b) Tumor volume from 'a' ($n=9$ /group); each line represents one mouse. (*) Based on a log-rank test and time to sacrifice (at 400mm^2) for analysis, mice treated with IL-2/mAb complexes had significantly improved outcomes versus each other condition ($p<0.001$ for each comparison). The average tumor areas when treatment was initiated ranged between $15\text{--}20\text{mm}^2$ between the 4 groups. (c) The frequency of donor Tc1 cells in the blood of mice ($n=4$ /group) treated as in 'a' but in the absence of tumor. Each point represents the average and bars indicate standard error. (d) The frequency of donor OT-I Tc1 cells in the blood of mice ($n=5$ /group) treated with mIL-2/mAb_{CD122} (clone S4B6) or mIL-2/mAb_{CD25} (clone 1A12). Each point represents the average and bars indicate standard error. (e) The frequency of donor polyclonal T cells in the blood of mice ($n=5$ /group) treated with hIL-2/mAb (clone 5355) complexes or vehicle alone. Each point represents the average and bars indicate standard error. For c-e, (**) indicates a significant difference ($p<0.001$) between indicated and other conditions. Random effects linear regression was used for modeling data and calculating p-values comparing conditions. All results are representative of at least 2 independent experiments.

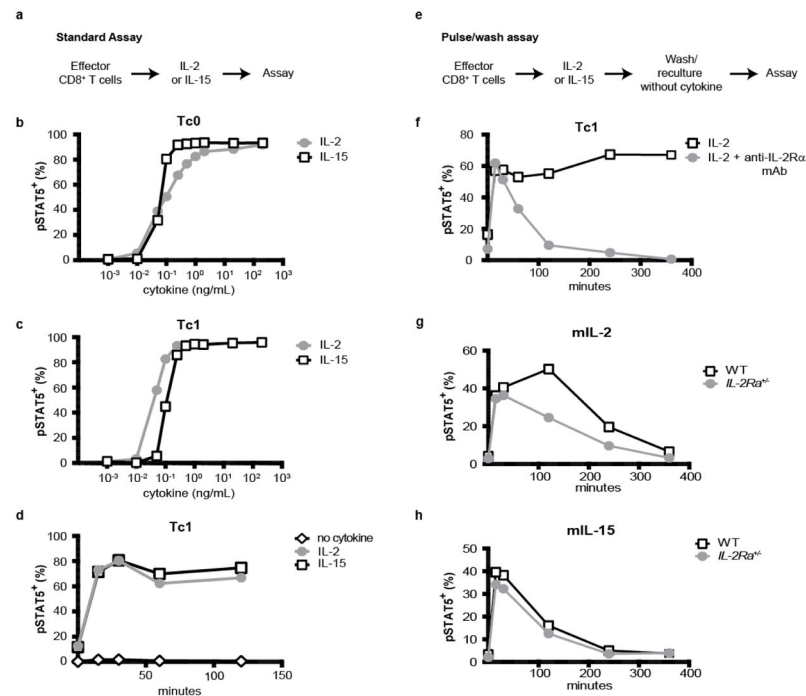


Figure 2. IL-2R α mediates sustained signaling in effector CD8⁺ T cells following withdrawal of IL-2

(a) Diagram of the standard cytokine assay in which effector cells are assayed after incubation with titrated cytokine. (b,c) Levels of pSTAT5 in Tc1 and Tc0 cells that were cultured with increasing amounts of mIL-2 or mIL-15 for 1 hour. (d) As in 'b', except Tc1 cells were incubated as indicated for up to 2 hours with 200ng/ml of cytokine and assayed for pSTAT5. (e) Diagram of the cytokine pulse assay in which effector cells are incubated with saturating amounts of cytokine (200ng/ml). Cells are then washed thoroughly, recultured at 37°C without additional cytokine, and assayed for pSTAT5. (f) Levels of pSTAT5 in Tc1 cells that were pulsed with mIL-2 with or without anti-IL-2R α mAb (PC61 clone) for 1 hour, washed, and recultured at 37°C for the times indicated. (g,h) Levels of pSTAT5 in polyclonal effector T cells from wildtype (IL-2R α ^{+/+}) or IL-2R α ^{+/-} mice that were pulsed for 1 hour with mIL-2 or mIL-15, and assayed as described in 'e'. Except for 'g' and 'h', all effector cells were generated from pmel-1 mice. All results are representative of at least 3 independent experiments.

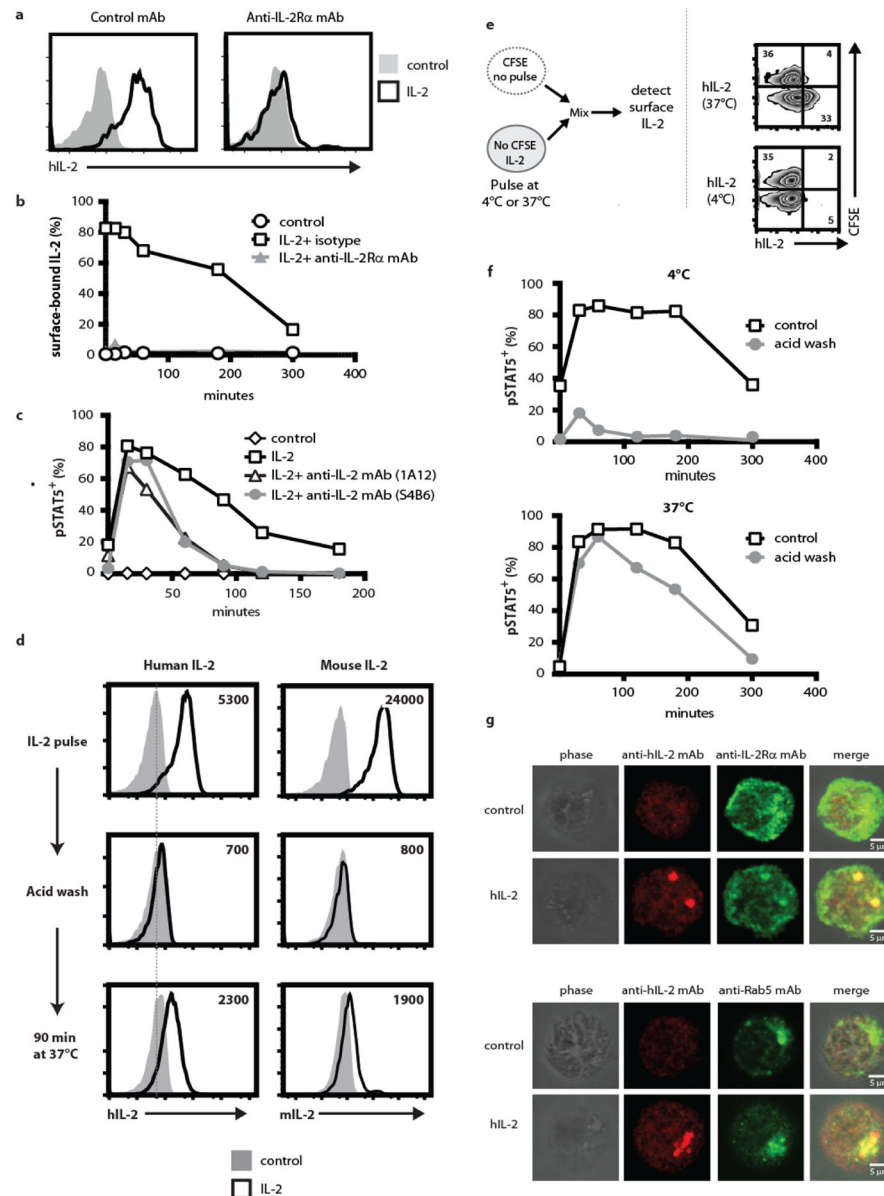


Figure 3. IL-2R α facilitates sustained IL-2 signaling through creation of an extracellular reservoir and recycling

(a) Presence of IL-2 on the surface of polyclonal T cells depends on IL-2R α . Polyclonal effector CD8⁺ T cells were pulsed for 2 hours with or without mIL-2. Prior to (and during) the pulse, T cells were incubated with anti-IL-2R α mAb (PC61). Cells were then washed and stained for surface IL-2. (b) Time course of surface IL-2 on polyclonal T cells after reculture at 37°C. (c) Levels of pSTAT5 in Tc1 cells that were pulsed with IL-2, washed, and recultured at 37°C with or without anti-IL-2 mAb (clone S4B6 or 1A12). (d) Recycling of IL-2 on effector T cells. Pmel-1 Tc1 cells were incubated with hIL-2 or mIL-2 at 37°C for 2 hours. As indicated, cells were then acid washed and recultured at 37°C for 90 minutes in the presence of anti-hIL-2 mAb conjugated to Alexa647. Cells were then washed, fixed, and assayed by flow cytometry. (e) Recycling of IL-2 on pulsed cells while mixed with non-

pulsed cells. Pmel-1 Tc1 cells were pulsed with hIL-2 at either 4°C or 37°C for 2 hours, and then acid washed. Cells were then mixed with non-pulsed CFSE-labeled Tc1 cells. The mixed cells were recultured at 37°C for 45 minutes in the presence of anti-hIL-2 mAb conjugated to Alexa647. Cells were then washed, fixed, and assayed by flow cytometry. (f) Internalized IL-2 leads to sustained pSTAT5 signaling. Pmel-1 Tc1 cells were pulsed with hIL-2 at either 4°C or 37°C for 2 hours, and then acid washed. Cells were then recultured at 37°C and assayed for pSTAT5. (g). Subcellular localization of hIL-2 and IL-2R α . Pmel-1 Tc1 cells were pulsed with hIL-2 (or media alone) for 1 hour at 37°C, and stained for hIL-2 and IL-2R α . Cells were then imaged by confocal microscopy. (h) Subcellular localization of hIL-2 and Rab5. As in 'g', except cells were stained for Rab5. Results are representative of 3 independent experiments.

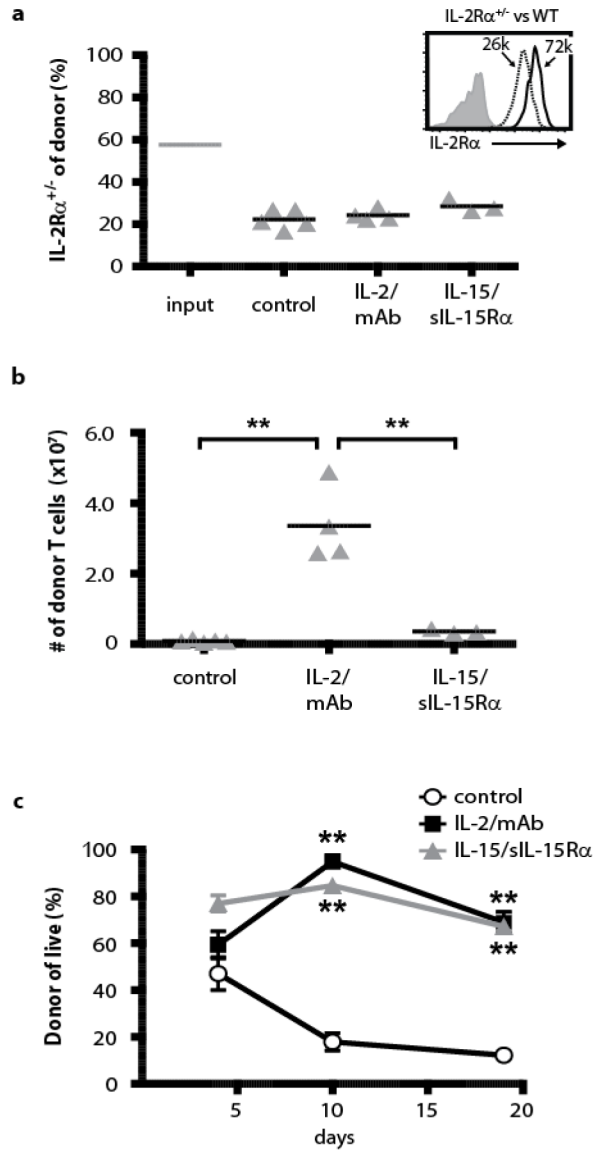


Figure 4. IL-2R α on donor T cells is critical for persistence in lymphoreplete but not lymphodepleted hosts

(a) Wildtype and IL-2R $\alpha^{+/-}$ effector CD8⁺ T cells show similar persistence with or without IL-2 therapy. Effector T cells from wildtype and IL-2R $\alpha^{+/-}$ mice were activated, mixed, and injected into recipient mice (n=3–5/group). Mice received injections of hIL-2/mAb (clone 5355) complexes, hIL-15/sIL-15R α complexes, or vehicle alone. The proportion of IL-2R $\alpha^{+/-}$ T cells among all donor T cells in the spleen was determined pre- and post-transfer. Each triangle represents one mouse and the bars indicate the mean. (b) The total number of donor T cells per spleen for the experiment shown in ‘a’. The bars indicate the mean. The symbol (**) indicates a significant difference (p<0.001) between indicated conditions. (c) In lymphodepleted mice, IL-2 and IL-15 therapy augment the persistence of effector Tc1 cells comparably. Pmel-1 Tc1 cells (10^7) were injected into mice given total body irradiation (600 rads) 1 day earlier. On days 0, 2, 4, and 6 after adoptive transfer, mice

(n = 5 per group) were treated with hIL-2/mAb (clone 5355) or hIL-15/sIL-15R α complexes, and the frequency of donor cells in blood was assayed at the time points indicated. Each point represents the average and bars indicate standard error. The symbol (**) indicates a significant difference ($p < 0.001$) between control and indicated conditions. All results are representative of 2 independent experiments.