IMMUNOTHERAPY

IL-2Rα mediates temporal regulation of IL-2 signaling and enhances immunotherapy

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Interleukin-2 (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-2Rα. IL-15 is under investigation as an alternative to IL-2. Although both cytokines signal through IL-2Rβγ, IL-15 does not bind IL-2Rα 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. Although 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-2Rα. Mechanistically, IL-2Rα 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-2Rα 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-2Rα expression on tumor-reactive lymphocytes may facilitate the development of more effective IL-2–based therapies.

INTRODUCTION

The administration of interleukin-2 (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-2Rα^{hi} T regulatory cells. IL-15 is a promising alternative. Like IL-2, IL-15 signals exclusively through the intermediate affinity IL-2Rβγ subunits (CD122/CD132). However, for high-affinity cytokine binding, IL-2 and IL-15 use specific IL-2Rα (CD25) and IL-15Rα 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-15Rα can lead to the recycling of IL-15, which sustains cellular signaling and lymphocyte survival (*7*). However, despite homology with IL-15Rα (*8*), IL-2Rα is not thought to facilitate sustained signaling or cytokine recycling because of lower affinity for IL-2 (*2–4*). Although briefly expressed on activated lymphocytes, IL-2Rα 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-2Rα develop T cell–mediated autoimmunity (*9*, *10*). In contrast, mice deficient in IL-15 or IL-15Rα are relatively healthy with reduced frequencies of CD8$^+$ memory-phenotype cells and natural killer (NK) cells (*11*, *12*). Therefore, given the potential undesirable consequences of engaging the IL-2Rα 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.

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RESULTS

IL-2 but not IL-15 therapy mediates antitumor 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 monoclonal antibody (IL-2/mAb) and IL-15/soluble IL-15Rα–Fc (IL-15/sIL-15Rα) 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 (subcutaneously) 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 T cell receptor (TCR) transgenic mice, from which CD8$^+$ T cells recognize an endogenous B16 tumor antigen (H-2D^b–restricted gp100$_{25–33}$ peptide). We have shown that these Tc1 effector cells are highly efficacious against tumor in lymphodepleted mice (*16*). For the first week after adoptive transfer, IL-15/sIL-15Rα or IL-2/mAb (clone 5355) complexes were administered every 48 hours. Whereas six of nine mice that received IL-2/mAb complexes were cured of established tumor, mice that received either IL-15/sIL-15Rα 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 or IL-15/sIL-15Rα 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 fig. S1A). Notably, without lymphodepletion or vaccination, we routinely achieved sustained donor T cell frequencies of 20% or higher in the

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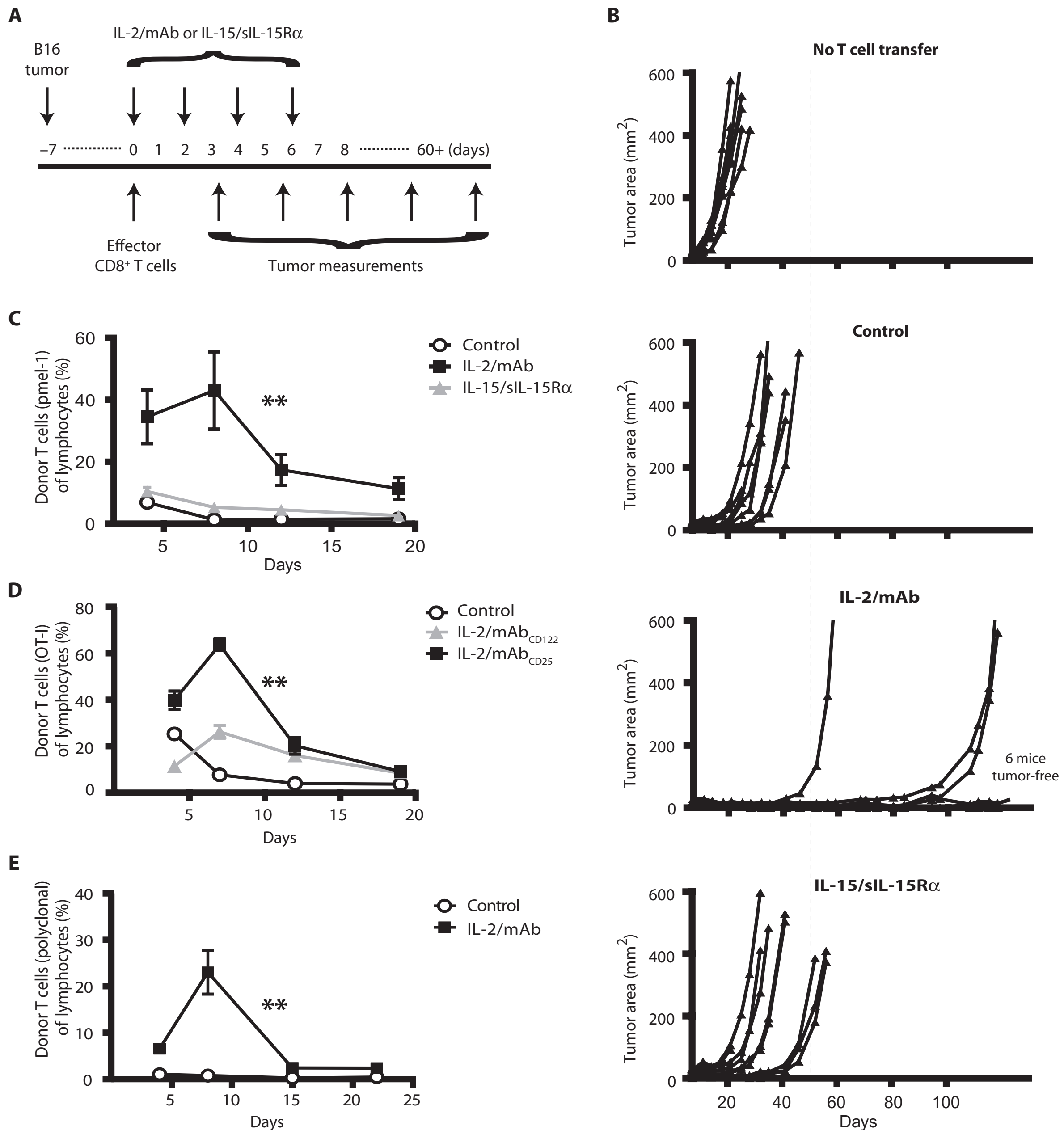

Fig. 1. IL-2/mAb but not IL-15/sIL-15Rα complexes induce potent effector T cell responses in tumor-bearing mice. (**A**) Treatment scheme for B6 mice injected subcutaneously with B16 melanoma tumor cells 7 days before the adoptive transfer of 3×10^6 pmel-1 Tc1 cells. Mice were then treated with human IL-2 (hIL-2)/mAb (clone 5355) or hIL-15/sIL-15Rα complexes. (**B**) Tumor volume from (A) (n = 9 per group); each line represents one mouse. On the basis of a log-rank test and time to sacrifice (at 400 mm^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 from 15 to 20 mm^2 between the four groups. (**C**) Frequency of donor Tc1 cells in the blood of mice (n = 4 per group) treated as in (A) but in the absence of tumor. Each point represents the average, and bars indicate SE. (**D**) Frequency of donor OT-I Tc1 cells in the blood of mice (n = 5 per group) treated with mouse IL-2 (mIL-2)/mAb_{CD122} (clone S4B6) or mIL-2/mAb_{CD25} (clone 1A12). Each point represents the average, and bars indicate SE. (**E**) Frequency of donor polyclonal T cells in the blood of mice (n = 5 per group) treated with hIL-2/mAb (clone 5355) complexes or vehicle alone. Each point represents the average, and bars indicate SE. **$P < 0.001$, significant difference between indicated and other conditions (C to E). Random effects linear regression was used for modeling data and calculating P values comparing conditions. All results are representative of at least two independent experiments.

peripheral blood. Furthermore, donor Tc1 cells were equally functional across treatment groups as indicated by the ability to produce interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α) (fig. S1B). 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-15Rα complexes exhibited minimally

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delayed tumor growth, albeit comparable between cytokine conditions (fig. S2).

Donor T cell expression of IL-2Rα 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-15Rα complexes was contrary to our expectation. This response was not dose-related because IL-2/mAb and IL-15/sIL-15Rα complexes expanded IL-2Rβγ^{hi} cells such as memory-phenotype CD8$^+$ T cells and NK cells to a similar extent in vivo (fig. S3) (*17*). However, only IL-2/mAb complexes expanded T regulatory cells (fig. S3), which are characterized by their expression of IL-2Rα. Because IL-12–conditioned (Tc1) effector CD8$^+$ T cells express very high levels of IL-2Rα (*16*), our results suggested an unappreciated role for cell surface IL-2Rα 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-2Rα expression. IL-2/mAb$_{CD25}$ complexes (clone 1A12) preferentially expand IL-2Rα^{hi} lymphocytes, whereas IL-2/mAb$_{CD122}$ complexes (clone S4B6) act in an IL-2Rα–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. Whereas 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-2Rα and not on IL-12 conditioning or selective TCR engagement, we stimulated polyclonal T cells from wild-type mice with plate-bound anti-CD3 mAb, a method that generates IL-2Rα^{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-2Rα (*16*), showed limited IL-2/mAb–driven persistence (fig. S4).

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

To uncover the mechanism behind the remarkable IL-2Rα–dependent responsiveness of effector Tc1 cells in vivo, we assayed IL-2 and IL-15 activity downstream of IL-2Rβγ using standard in vitro assays quantifying phosphorylation of STAT5 (signal transducer and activator of transcription 5; 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-2Rα^{hi}) cells exhibited marginally increased sensitivity to IL-2 versus IL-15 when compared to Tc0 effector cells (IL-2Rα^{med}) (Fig. 2, B and C), which is consistent with previous findings (*19*). The addition of a blocking antibody (anti–IL-2Rα mAb, PC61 clone) also showed a minimal benefit of IL-2Rα engagement on Tc1 cells in comparison between titrated IL-2 and IL-15 (fig. S5). Notably, Tc1 cells responded comparably to IL-2 and IL-15 in standard assays of proliferation and viability (fig. S6). 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 marked difference in activity observed in vivo. Therefore, we hypothesized that IL-2Rα 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 Fig. 2E. Consistent with our hypothesis, only preculture of Tc1 cells with IL-2 led to sustained STAT5 phosphorylation in the absence of additional cytokine (fig. S7). To directly test the role of IL-2Rα in promoting sustained signaling on effector CD8$^+$ T cells, we cultured Tc1 cells for 90 min with IL-2 in the absence or presence of blocking anti–IL-2Rα antibody (PC61 clone). This shorter pulse was equally sufficient for inducing sustained signaling as indicated by STAT5 phosphorylation (Fig. 2F). Blockade of IL-2Rα completely abolished the sustained IL-2 signaling as indicated by STAT5 phosphorylation and proliferation (Fig. 2F and figs. S8 and S9). Polyclonal effector CD8$^+$ T cells activated in the absence of IL-12 also showed sustained IL-2 signaling, and effector cells generated from IL-2Rα$^{+/-}$ mice showed roughly half the sustained IL-2 signaling (Fig. 2G). To ensure that these cells had similar IL-2Rβγ signaling potential, we pulsed wild-type and IL-2Rα$^{+/-}$ 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 (fig. S10). Furthermore, culture of human effector T cells with hIL-2 but not hIL-15 led to IL-2Rα–dependent sustained STAT5 phosphorylation (fig. S11). Finally, to verify that IL-2/mAb complexes (clone 5355) used in our in vivo experiments were permissive to engagement of IL-2Rα, 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-2Rα (fig. S12A). In contrast, IL-2/mAb$_{CD122}$ complexes (clone S4B6), which do not engage IL-2Rα (*15*, *18*), failed to induce sustained signaling in vitro (fig. S12B).

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

To understand how IL-2Rα promotes sustained IL-2 signaling, we hypothesized two non–mutually exclusive possibilities. First, IL-2Rα may bind IL-2 and create a cell surface cytokine reservoir due to the high ratio of surface IL-2Rα to IL-2Rβγ, because IL-2/IL-2Rα internalization can only occur in the presence of both IL-2Rβ and IL-2Rγ (*20*, *21*). Such a reservoir of IL-2 bound to IL-2Rα would mediate gradual signaling by continually feeding the rate-limiting, endocytosed IL-2Rβγ. 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-2Rα (Fig. 3, A and 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-2Rα 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 reappearance 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). The species specificity of our reagents precluded autocrine production as the source of cell surface IL-2 after acid wash (fig. S13). In additional support of IL-2Rα–mediated recycling, we observed sustained pSTAT5 signaling after acid washing of cells pulsed with hIL-2 at 37°C but not at 4°C (Fig. 3F). Because internalization of IL-2Rαβγ does not occur at 4°C, these data provide further support that sustained signaling occurs in part through an IL-2Rα–bound pool of internalized IL-2. It is notable that we could not block

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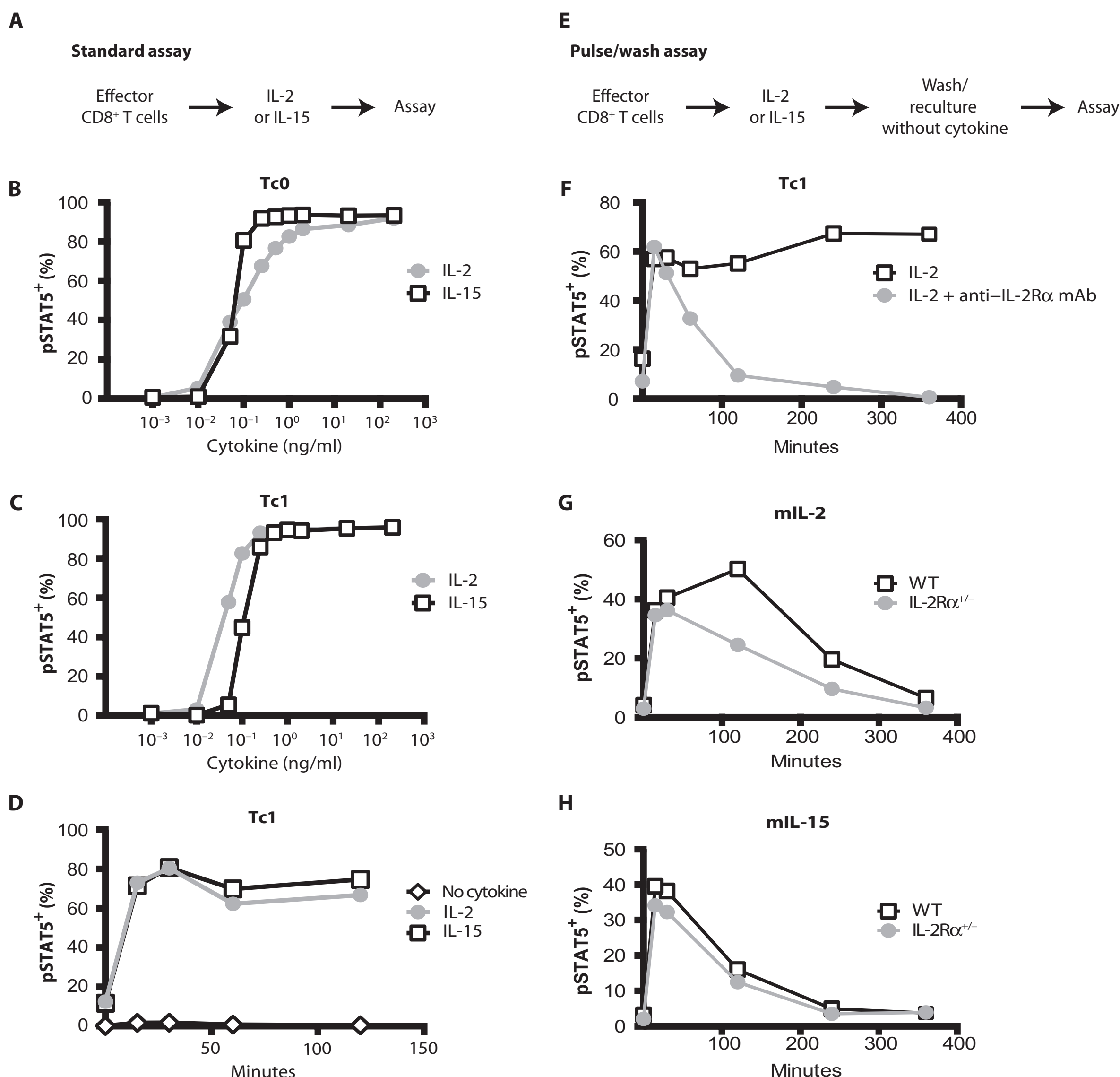

Fig. 2. IL-2Rα mediates sustained signaling in effector CD8$^+$ T cells after withdrawal of IL-2. (**A**) Diagram of the standard cytokine assay in which effector cells are assayed after incubation with titrated cytokine. (**B** and **C**) Levels of phosphorylated STAT5 (pSTAT5) in Tc1 and Tc0 cells 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 cytokine (200 ng/ml) and assayed for pSTAT5. (**E**) Diagram of the cytokine pulse assay in which effector cells are incubated with saturating amounts of cytokine (200 ng/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-2Rα mAb (PC61 clone) for 1 hour, washed, and recultured at 37°C for the times indicated. (**G** and **H**) Levels of pSTAT5 in polyclonal effector T cells from wild-type (WT) (IL-2Rα$^{+/+}$) or IL-2Rα$^{+/-}$ 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 three independent experiments.

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-2Rα compared with that of hIL-2 for mIL-2Rα (*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 at 4°C (figs. S14 and S15). These punctate structures colocalized with IL-2Rα, Rab5, and EEA1 (early endosome antigen 1), but less frequently with LAMP-1 (lysosomal-associated membrane protein 1), consistent with intracellular IL-2 being accessible to the recycling pathway (Fig. 3, G and H, and fig. S16) (*23*, *24*). Together, these results suggest that IL-2Rα both promotes an extracellular reservoir for IL-2 and mediates recycling of IL-2.

IL-2Rα 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-2Rα on

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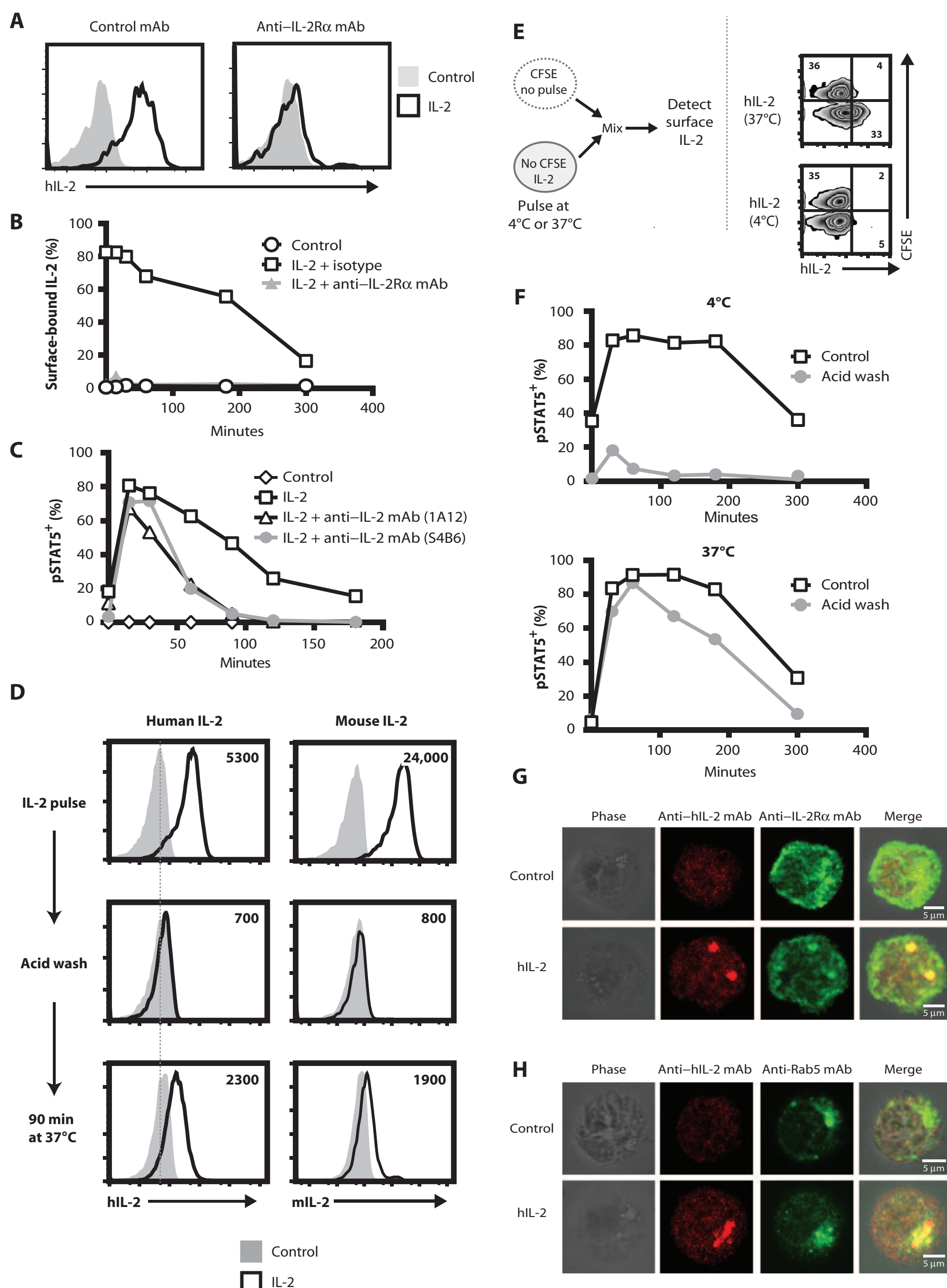

Fig. 3. IL-2Rα facilitates sustained IL-2 signaling through creation of an extracellular reservoir and recycling. (**A**) The presence of IL-2 on the surface of polyclonal T cells depends on IL-2Rα. Polyclonal effector $CD8^+$ T cells were pulsed for 2 hours with or without mIL-2. Before (and during) the pulse, T cells were incubated with anti–IL-2Rα 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 min in the presence of anti–hIL-2 mAb conjugated to Alexa 647. Cells were then washed, fixed, and assayed by flow cytometry. (**E**) Recycling of IL-2 on pulsed cells while mixed with nonpulsed 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 nonpulsed carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled Tc1 cells. The mixed cells were recultured at 37°C for 45 min in the presence of anti–hIL-2 mAb conjugated to Alexa 647. 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-2Rα. Pmel-1 Tc1 cells were pulsed with hIL-2 (or medium alone) for 1 hour at 37°C and stained for hIL-2 and IL-2Rα. 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 three independent experiments.

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donor T cells providing a competitive advantage to accessing cytokine. To formally test this hypothesis, we initially attempted to activate T cells from wild-type and IL-2Ra$^{-/-}$ mice. However, this proved technically not feasible for us because T cells isolated from IL-2Ra$^{-/-}$ mice were resistant to normal activation, likely because of the immune alterations in the absence of IL-2 responsiveness (*9*). Therefore, we used polyclonal IL-2Rα$^{+/-}$ T cells because these cells activated comparably to wild-type T cells and had about half the expression of IL-2Rα (Fig. 4A). Using the Thy1.1 congenic marker to distinguish between genotypes, we mixed these two cell populations and adoptively transferred them into nonirradiated B6(CD45.1) recipient mice. Mice were treated with IL-2/mAb or IL-15/sIL-15Rα for 1 week. We hypothesized that IL-2Rα$^{+/-}$ donor CD8$^+$ T cells would not persist as well as their wild-type counterparts because of loss of one allele. In contrast to our expectations, wild-type and IL-2Rα$^{+/-}$ donor T cells did not show differential responsiveness to treatment with IL-2/mAb or IL-15/sIL-15Rα complexes (Fig. 4, A and B). These results suggest a threshold of IL-2Rα in vivo, in terms of both 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-2Rα on donor T cells in vivo, we compared the responsiveness of IL-2Rα^{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-2Rα–competent cytokine therapy would be lost in the absence of host IL-2Rβγ$^+$ lymphocytes competing for cytokine (fig. S17A). Thus, mice were given total body irradiation (600 rads) before adoptive transfer of effector Tc1 CD8$^+$ T cells and then treated for 1 week with IL-2/mAb and IL-15/sIL-15Rα 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 fig. S17B). These results demonstrate a critical role for IL-2Rα on donor T cells in promoting IL-2 responsiveness in a lymphoreplete host environment.

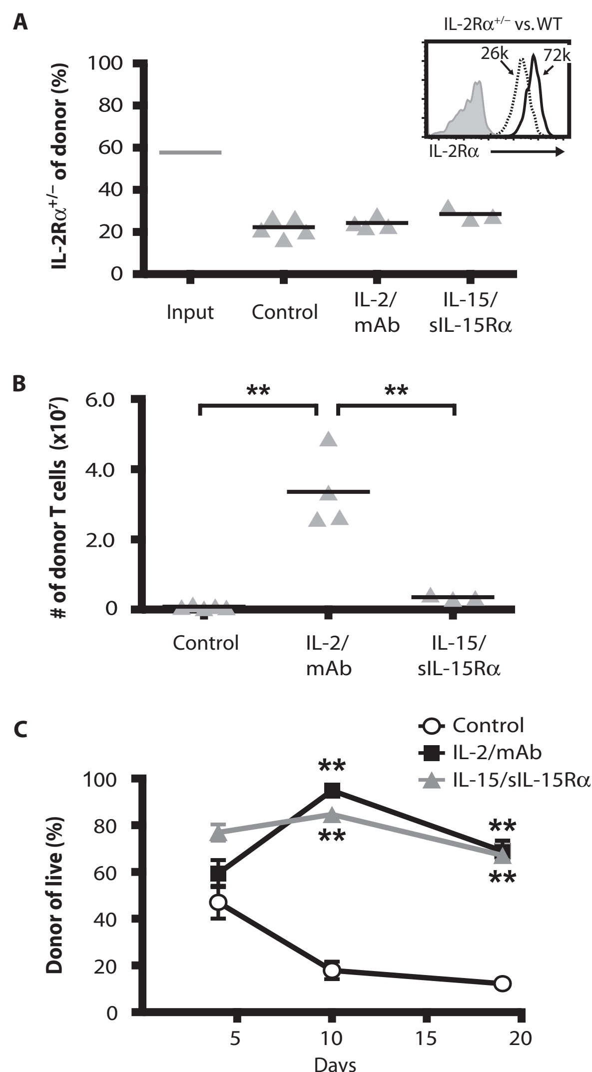

Fig. 4. IL-2Rα on donor T cells is critical for persistence in lymphoreplete but not lymphodepleted hosts. (**A**) WT and IL-2Rα$^{+/-}$ effector CD8$^+$ T cells show similar persistence with or without IL-2 therapy. Effector T cells from WT and IL-2Rα$^{+/-}$ mice were activated, mixed, and injected into recipient mice (n = 3 to 5 per group). Mice received injections of hIL-2/mAb (clone 5355) complexes, hIL-15/sIL-15Rα complexes, or vehicle alone. The proportion of IL-2Rα$^{+/-}$ T cells among all donor T cells in the spleen was determined before and after transfer. Each triangle represents one mouse, and bars indicate the mean. (**B**) Total number of donor T cells per spleen for the experiment shown in (A). Bars indicate the mean. $^{**}P < 0.001$, significant difference between indicated conditions. (**C**) Total number of donor T cells in the spleen for the experiment in (A). Mice (n = 5 per group) were given total body irradiation (600 rads) 1 day before adoptive transfer of 1×10^7 Tc1 (pmel-1) cells and then treated with hIL-2/mAb (clone 5355) or hIL-15/sIL-15Rα complexes. The frequency of donor T cells in the blood of mice was determined at the indicated time points. Each point represents the average, and bars indicate SE. $^{**}P < 0.001$, significant difference between control and indicated conditions. All results are representative of two independent experiments.

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, because 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-2Rα–competent cytokine therapy led to sustained persistence and antitumor immunity of effector CD8$^+$ T cells expressing elevated IL-2Rα. Host cells appear to play an important role in this process, because in mice preconditioned with total body irradiation, IL-2Rα–independent (or IL-15–based) cytokine therapy effectively augmented donor T cell responses. Together, these results suggest that IL-2Rα 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 because 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 that explain how IL-2Rα increases the durability of IL-2 signaling. It has been widely assumed that IL-2Rα improves IL-2 responsiveness primarily by acting as a high-affinity receptor. We find, however, that beyond ligand affinity, IL-2Rα can promote sustained IL-2 signaling after withdrawal of cytokine, thereby providing

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a temporal dimension to IL-2 signaling. To enable sustained signaling, we find at least two distinct IL-2Rα–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-2Rα subunit is recycled after cytokine-mediated internalization, instead of being degraded like the IL-2Rβγ subunits (*25*), our results suggest a mechanism by which IL-2Rα can rescue IL-2 from degradation. These properties may be relevant during early stages of infection when lymphocytes express high levels of IL-2Rα (*26*–*28*).

Our results with IL-2Rα are reminiscent of the ability of membrane-bound IL-15Rα to facilitate recycling and sustained cellular responsiveness to IL-15 (*7*). Mutants of IL-2, selected for high-affinity binding to IL-2Rα (and thus thought to more resemble IL-15 in terms of high affinity for cognate α 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*). Our findings are distinct because they show that wild-type IL-2 can induce sustained cytokine signaling on primary mouse and human lymphocytes. Furthermore, we find that sustained IL-2 signaling is directly dependent on IL-2Rα as shown by antibody-mediated blockade and genetic targeting.

There are emerging questions from our study that warrant additional investigation. Foremost, our results suggest that strategies to enhance IL-2Rα expression on donor T cells may allow for more effective IL-2–based antitumor responses. In this context, it will be important to assess the therapeutic potential of strategies that redirect IL-2 toward IL-2Rα, 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-2Rα affect 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-2Rα–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-2Rα 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 adenosine diphosphate ribosylation can regulate IL-2 binding to IL-2Rα (*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 nonlymphopenic patients, IL-2Rα–competent cytokine therapy will be effective if responding T cells express elevated levels of IL-2Rα. In contrast, in lymphodepleted patients where host cells are destroyed and do not compete for cytokine availability, IL-2Rα–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 antitumor 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, we 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. Please also see table S1 for raw data.

Recombinant proteins and antibodies

hIL-15, hIL-2, and anti-hCD3 mAb (clone OKT3) were provided by the National Cancer Institute Biological Resources Branch Preclinical Repository. mIL-2, mIL-12, and mIL-15 were purchased from Shenandoah Biotechnology. Recombinant sIL-15Rα–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 Biosciences. Anti–mIL-2 mAb_{CD122} (clone S4B6) and anti–IL-2Rα (clone PC61) were obtained from Bio X Cell. 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 University of California, San Francisco, 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 the 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 the American Type Culture Collection.

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 3 days with relevant peptide [for pmel-1, $hgp100_{25-33}$ peptide (KVPRNQDWL) and for OT-I, $OVA_{257-264}$ peptide (SIINFEKL)] and cultured with (Tc1) or without (Tc0) mIL-12 (10 ng/ml). Polyclonal mouse T cells were generated by culturing B6 splenocytes for 3 days with plate-bound anti-CD3 mAb (clone 145-2C11; 1 μg/ml) unless otherwise stated. Activated human T cells were generated by culturing de-identified human peripheral blood mononuclear cells (Research Blood Components) from healthy adult donors for 2 or 3 days with plate-bound anti-CD3 mAb (clone OKT3; 1 μg/ml).

Tumor and persistence studies in mice

For tumor experiments, B6 mice were challenged subcutaneously with 2.5×10^5 B16-F1 tumor cells. Before randomizing mice to treatment groups, some mice were excluded because of 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 the following: hIL-15/sIL-15Rα, hIL-15 (0.5 μg)/sIL-15Rα–Fc (2.3 μg); hIL-2/mAb, hIL-2 (1.5 μg)/anti–IL-2 mAb (7.5 μg; clone 5355); hIL-2/mAb_{CD25}, hIL-2 (1.5 μg)/anti–IL-2 mAb (7.5 μg; clone 5344.111); mIL-2/mAb_{CD122}, mIL-2 (1.5 μg)/anti–IL-2 mAb (7.5 μg; clone S4B6); and mIL-2/mAb_{CD25}, mIL-2 (1.5 μg)/anti–IL-2 mAb (7.5 μg; clone JES6-1A12). Tumor growth was measured by caliper every 2 to 4 days by personnel blinded to the treatment regimen. Tumor surface area (mm^2) was calculated as length × width. Mice were

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sacrificed when tumors reached 400 mm^2. 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 wild-type (Thy1.1) and IL-2R$\alpha^{+/-}$ (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 (600 rads) 1 day before 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 Biosciences, BioLegend, and eBioscience. For analysis of phosphorylation of STAT5, we followed the manufacturer's protocol using Lyse/Fix Buffer and Perm Buffer III (BD Biosciences). To examine cellular proliferation, cells were fixed and permeabilized according to the manufacturer's protocol for Cytofix/Cytoperm (BD Biosciences) and stained with anti-Ki67 mAb (SolA15, eBioscience). Alternatively, 5-bromo-2′-deoxyuridine (BrdU; 10 μM) was added 1 hour before 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 LSR II and BD FACS Accuri. Data were analyzed using FlowJo software (Tree Star). In all experiments, initial gating of live cells was performed using forward 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, or propidium iodide. For pulse assays, cells were incubated with or without cytokine at 200 ng/ml at either 4°C or 37°C for 90 min 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-2Rα mAb was added 15 min before cytokine addition. Acid wash was performed by washing cells twice for 2 min at 4°C with an acid wash buffer consisting of complete medium adjusted to pH 3.5 or pH 3.75 with 1 N HCl. For analysis of recycling of IL-2 to the cell surface, acid-washed cells were replated in medium at 37°C for the indicated amount of time with anti–IL-2 mAb conjugated to Alexa 647 (5334 or 5B9-2-1 clone). To assess IFN-γ and TNF-α production, we added $hgp100_{25-33}$ (1 μg/ml) or phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 μM) to splenocytes for 6 hours in the presence of brefeldin A (GolgiStop, BD Biosciences).

Confocal microscopy

Tc1 cells were incubated with hIL-2 (200 ng/ml), mIL-2 (200 ng/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 Cytofix/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-2Rα polyclonal antibody (R&D Systems), anti-Rab5 mAb (C8B1, Cell Signaling), anti–LAMP-1 mAb (1D4B, BioLegend), or anti-EEA1 mAb (C45B10, Cell Signaling). To detect anti–IL-2Rα, we used an anti-goat immunoglobulin G (IgG) conjugated to Alexa 488 (R&D Systems). To detect EEA1 and Rab5, we used an anti-rabbit IgG conjugated to Alexa 488 [$F(ab')_2$ 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) using a 60× water immersion objective (1.2 numerical aperture). 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 versus 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 were 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 time points 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 (for example, % 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. Percent colocalization was compared with log(percent) as the outcome (due to skewness) and main effects of LAMP-1 (versus EEA1) and rater. The LAMP-1 effect was evaluated on the basis of the Wald test of the regression coefficient. Model results were exponentiated to provide point estimates for LAMP-1 and EEA1 colocalization. In the interest of addressing our hypotheses and not overtesting, 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 α level of 0.05. Statistical analyses were performed using Stata/IC (version 12.1) and R statistical software.

SUPPLEMENTARY MATERIALS

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Fig. S1. IL-2/mAb complexes selectively enhance the persistence of donor T cells.

Fig. S2. In the absence of donor T cells, hIL-2/mAb and IL-15/sIL-15Rα complexes mediate comparable antitumor immunity.

Fig. S3. Treatment with IL-2/mAb, IL-2/mAb_{CD25}, and IL-15/sIL-15Rα complexes induces differential expansion of $CD8^+$ memory-phenotype T cells, NK cells, and T regulatory cells.

Fig. S4. Tc1 but not Tc0 effector $CD8^+$ T cells show preferential responsiveness to IL-2/mAb complexes.

Fig. S5. Blockade of IL-2Rα has minimal impact on Tc1 cells in response to titrated IL-2 or IL-15.

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Fig. S6. Tc1 effector CD8⁺ T cells exhibit comparable functional sensitivity to IL-2 and IL-15 in vitro.
Fig. S7. Tc1 effector CD8⁺ T cells pulsed with IL-2 mediate sustained cytokine signaling.
Fig. S8. IL-2–mediated sustained cytokine signaling is IL-2Rα–dependent in 11 independent experiments.
Fig. S9. Tc1 effector CD8⁺ T cells pulsed with IL-2 exhibit IL-2Rα–dependent proliferation after cytokine withdrawal.
Fig. S10. Human IL-2 mediates sustained cytokine signaling on mouse Tc1 effector CD8⁺ T cells.
Fig. S11. Human effector CD8⁺ T cells pulsed with IL-2 mediate sustained IL-2Rα–dependent signaling.
Fig. S12. Human IL-2/mAb (clone 5355), but not mouse IL-2/mAb$_{CD122}$ (clone S4B6), complexes are permissive to IL-2Rα–dependent sustained signaling in vitro.
Fig. S13. Antibodies for mouse and human IL-2 are species-specific.
Fig. S14. Detection of hIL-2 by confocal microscopy is species-specific and dependent on pulsing cells with cytokine at 37°C.
Fig. S15. Detection of mIL-2 by confocal microscopy.
Fig. S16. Colocalization of hIL-2 with EEA1 and LAMP-1 by confocal microscopy.
Fig. S17. In lymphodepleted mice, IL-15/sIL-15Rα and hIL-2/mAb mediate comparable engraftment of Tc1 effector CD8⁺ T cells.
Table S1. Raw data for main and supplementary figures.

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IL-2Rα mediates temporal regulation of IL-2 signaling and enhances immunotherapy

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Immunotherapy: Reduce, reuse, recycle
Interleukin-2 (IL-2) is a growth factor used in the clinic to boost immune cell responses to cancer. The related cytokine IL-15 also expands conventional T cells but, unlike IL-2, does not expand regulatory T cells. Now, Su *et al.* demonstrate that although IL-15 may be effective in lymphopenic patients, IL-2 therapy may be needed in patients who retain immune cells, where T cells compete for available cytokine. The authors show that IL-2 but not IL-15 can sustain signaling after cytokine withdrawal through its high-affinity receptor subunit IL-2Rα. IL-2Rα forms a cell surface reservoir of IL-2 and also aids in recycling IL-2 back to the cell surface, increasing the efficiency of limited quantities of cytokine.

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