

IMMUNOLOGY

IL-2R β abundance differentially tunes IL-2 signaling dynamics in CD4⁺ and CD8⁺ T cellsGeoffrey A. Smith,^{1,2} Jack Taunton,¹ Arthur Weiss^{2,3*}

Interleukin-2 (IL-2) stimulates both activated CD4⁺ and CD8⁺ T cells to proliferate. IL-2 signals through an identical receptor complex and promotes the same dose-dependent phosphorylation of the canonical transcription factor STAT5 in both cell types. Despite this, CD8⁺ T cells enter the S phase earlier and proliferate to a greater extent than do CD4⁺ T cells in response to IL-2. We identified distinct IL-2 signaling dynamics in CD4⁺ and CD8⁺ T cells. In IL-2-stimulated CD8⁺ T cells, STAT5 phosphorylation increased rapidly and was sustained for 6 hours. In contrast, CD4⁺ T cells had a biphasic response, with maxima at 15 min and 2 to 4 hours after stimulation. Both cell types required vesicular trafficking, but only CD4⁺ T cells required new protein synthesis to maintain high phosphorylation of STAT5. Two subunits of the IL-2 receptor, IL-2R β and IL-2R γ , were twice as abundant in CD8⁺ T cells than in CD4⁺ T cells. Reduction of IL-2R β abundance by 50% was sufficient to convert CD8⁺ T cells to a CD4⁺ T cell–like signaling pattern and delay S phase entry. These results suggest that the larger pool of IL-2R β chains in CD8⁺ T cells is required to sustain IL-2 signaling and contributes to the quantitatively greater proliferative response to IL-2 relative to that of CD4⁺ T cells. This cell type–specific difference in IL-2R β abundance appears to tune responses, potentially preventing extensive, autoimmune proliferation of CD4⁺ T cells, while still enabling sufficient proliferation of CD8⁺ T cells to control viral infections.

INTRODUCTION

The common γ chain cytokine interleukin-2 (IL-2) plays an essential role in the development, expansion, and function of many lymphocyte subsets (1). IL-2 induces signaling by binding to a receptor complex minimally composed of the IL-2R β and IL-2R γ subunits. Binding induces a conformational change that activates the associated cytoplasmic kinases Janus kinase 1 (JAK1) and JAK3, which in turn phosphorylate three tyrosine residues on the IL-2R β chain. Phosphorylated Tyr³⁵² and Tyr⁵¹⁰ recruit the transcription factor signal transducer and activator of transcription 5 (STAT5), which is rapidly phosphorylated and alters transcription of IL-2–dependent genes (2). Phosphorylated Tyr³³⁸ recruits the adapter protein Shc, which can activate phosphoinositide 3-kinase (PI3K) signaling and, in some cell types, mitogen-activated protein kinase (MAPK) signaling. Depending on the cell type and physiologic context, these signals combine to trigger proliferation, production of effector molecules, or differentiation into distinct T cell subsets, such as regulatory T cells (T_{regs}) or memory T cells.

IL-2–driven cell fate changes require hours to days of sustained signaling. Differentiation into T_{regs} requires up to 3 days of IL-2 exposure (3), and S phase entry by effector T cells requires 8 to 12 hours of continuous IL-2 stimulation (4, 5). Despite this, most studies of IL-2 signaling have focused on events immediately after the onset of stimulation. In our previous work in activated CD4⁺ T cells, we examined IL-2 signaling throughout this critical 8- to 12-hour period before S phase entry and found that there are two waves of STAT5 phosphorylation (pSTAT5) after stimulation: an initial strong, rapid induction of pSTAT5 that peaks within 15 min and decays within an hour and a second wave, which appears between 2 and 12 hours after the onset of stimulation. Abolishing the second wave of pSTAT5 was sufficient to prevent S phase entry (5). This unique signaling pattern, coupled

with the importance of sustained IL-2 signaling, raises the question of how signaling dynamics vary by cell type or cytokine and how this impacts cell fate decisions.

At physiologic doses, IL-2 signals through the “high-affinity” [K_d (dissociation constant), ~ 10 pM] receptor complex composed of IL-2R α (CD25), IL-2R β (CD122), and IL-2R γ (CD132) (6). This trimeric architecture enables regulation of T cell IL-2 responsiveness at multiple levels. Whereas all T cells express IL-2R γ , resting naive T cells do not express IL-2R α and are unresponsive to physiologic doses of IL-2 (7). Upon activation of these cells through the T cell receptor (TCR), IL-2R α is induced to very high abundance, at least 10-fold higher than IL-2R β or IL-2R γ (8–10), and cells become sensitive to IL-2. Additionally on naive CD4⁺ T cells, surface abundance of IL-2R β is at or below the limit of detection by fluorescence-activated cell sorting (FACS), rendering these cells minimally responsive to IL-2 until at least 24 hours after TCR activation (11). By contrast, naive CD8⁺ T cells have a small, but detectable, quantity of IL-2R β chains and can respond to high doses of IL-2 through the “intermediate-affinity” (K_d , ~ 1 nM) receptor (6, 12). Upon IL-2 binding, receptor complexes are rapidly internalized [surface $t_{1/2}$ (half-life), ~ 15 min], and most of the IL-2R β and IL-2R γ subunits are degraded ($t_{1/2}$, ~ 50 to 70 min), whereas IL-2R α is recycled to the surface (degradation $t_{1/2}$, >6 hours) (13, 14).

Given the requirement for sustained IL-2 signaling and the short half-life of IL-2–bound receptor complexes (15), the precise number and subcellular localization of individual IL-2 receptor subunits is a potentially important regulatory mechanism. Fiftyfold overexpression of the epidermal growth factor receptor in a neuronal cell line converts a transient pulse of extracellular signal–regulated kinase (ERK) signaling into a sustained signal that triggers cell differentiation (16). A threshold for sustained TCR signaling allows two- to threefold differences in receptor abundance to translate into binary outcomes (17) and provides a check on hyperresponsive T cells (18). However, in the IL-2R system, the relationship between receptor abundance, dynamics, and cell fate is not known.

Here, we compared IL-2 signaling dynamics in mouse CD4⁺ and CD8⁺ T cells. As others have reported (19), we found that IL-2 triggered

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a quantitatively stronger proliferative response in CD8⁺ T cells compared to CD4⁺ T cells. Unlike the biphasic response seen in CD4⁺ T cells, CD8⁺ T cells had a very different pattern: a strong and sustained induction of pSTAT5. We compared the abundance of IL-2R subunits and found that CD8⁺ T cells expressed about twice as much IL-2Rβ and IL-2Rγ as CD4⁺ T cells. By examining the dynamics of other cytokines that, like IL-2, signal through the common γ chain (γ_c, IL-2Rγ_c), we identified IL-2Rβ as a limiting receptor component in CD4⁺ T cells. A 50% reduction of IL-2Rβ abundance in CD8⁺ T cells converted their signaling dynamics to a CD4⁺-like phenotype and greatly blunted their proliferative response. These results suggest a model whereby IL-2Rβ abundance across a narrow range can markedly alter the IL-2 responsiveness of a cell.

RESULTS

CD8 T cells are hyperproliferative in response to IL-2

Because naïve T cells do not express the high-affinity IL-2 receptor, we generated CD4⁺ and CD8⁺ T cell blasts from primary murine T cells to compare IL-2 signaling in each cell type. Upon stimulation with a physiologic dose of IL-2 (50 U/ml), both resting CD4⁺ and CD8⁺ T cell blasts enter the S phase and proliferate. However, the CD8⁺ T cell blasts entered the S phase sooner and more synchronously than did the CD4⁺ T cell blasts (Fig. 1A). Consequently, CD8⁺ T cell blasts completed more rounds of cell division in 72 hours under continuous IL-2 stimulation (Fig. 1B). This increased proliferative response of CD8⁺ versus CD4⁺ T cells to IL-2 has been reported previously (19). Furthermore, enhanced CD8⁺ versus CD4⁺ proliferation has also been observed in response to simultaneous TCR and IL-2 stimulation *in vitro* and *in vivo* (20, 21).

CD4⁺ and CD8⁺ T cells have distinct IL-2 signaling dynamics

To understand the signaling basis for this enhanced response in CD8⁺ T cell blasts, we first assessed the immediate ($t = 15$ min) phosphorylation of STAT5, which is essential for IL-2–driven proliferation (2). In response to a wide titration of IL-2 concentrations, CD4⁺ and CD8⁺

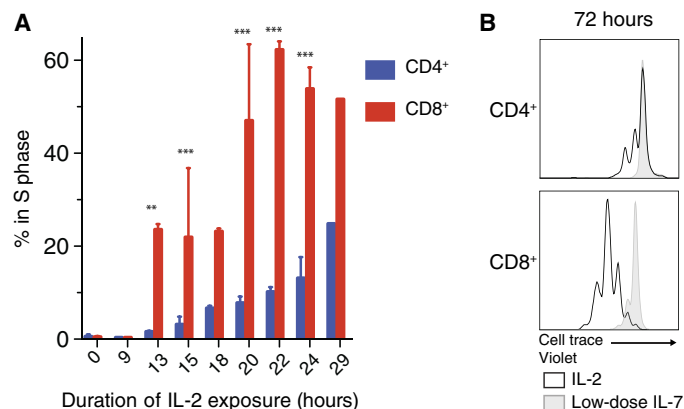


Fig. 1. Proliferative responses of CD4 and CD8 T cells to IL-2. (A) S phase entry of rested CD4⁺ and CD8⁺ T cell blasts was assessed with a 1-hour pulse of 5-ethynyl-2'-deoxyuridine (EdU) after the indicated duration of interleukin-2 (IL-2) stimulation. (B) Cell proliferation after 72 hours of IL-2 stimulation was assessed by dye dilution (CellTrace Violet) compared to undivided cells sustained with low-dose IL-7. Pooled results from seven independent experiments plotted as means \pm SEM (A) or representative of three independent experiments (B). Time course (A) compared by repeated-measures analysis of variance (ANOVA) (cell type, time, and interaction, all $P < 0.0001$) with Bonferroni multiple comparison test (significant $P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ for CD4s versus CD8s at a given time point).

T cell blasts displayed nearly identical dose responses [EC_{50} (half maximal effect concentration), 0.35 and 0.28 U/ml, respectively; Fig. 2, A and B]. At high IL-2 concentrations, the magnitude of the response (mean fluorescence intensity of the responding population) was about 25% higher in CD8⁺ T cells compared to CD4⁺ T cells (fig. S1, A and B). Although it was tempting to attribute the enhanced proliferation of CD8⁺ T cells to this increase in maximal pSTAT5, the requirement for 8 to 12 hours of continuous IL-2 exposure led us to suspect a more complicated mechanism (4, 5).

In CD4⁺ T cells, IL-2 triggers a biphasic STAT5 phosphorylation response, with an initial peak of phosphorylation 15 to 30 min after the onset of IL-2 stimulation and a second peak at 2 to 12 hours of stimulation (5). This prompted us to ask whether CD8⁺ T cells displayed a similar pattern of STAT5 phosphorylation (pSTAT5). To our surprise, CD8⁺ and CD4⁺ T cells exhibited very different signaling dynamics (Fig. 2, C and D). Unlike CD4⁺ T cells, there was a single, sustained peak of pSTAT5 in CD8⁺ T cells without the characteristic decrease in phosphorylation seen at 1 hour in CD4⁺ T cells. The fraction of pSTAT5⁺ cells was significantly higher in CD8⁺ T cells at 45 min, 1 hour, and 2 hours after the onset of IL-2 stimulation but similar before and after those time points. These patterns persisted at a range of physiologic IL-2 concentrations from 1 to 250 U/ml (fig. S1, C and D).

To understand whether differences in the first 2 hours of signaling could contribute to the CD8⁺ hyperproliferative phenotype, we examined the protein product of the immediate-early gene *Myc*, induction of which is essential for cell cycle progression. Studies have shown that both CD4⁺ and CD8⁺ T cells require a minimum threshold of Myc protein to divide (11, 22). Within the first 2 hours of IL-2 stimulation, the Myc protein increased almost 4-fold over baseline in CD8⁺ T cells compared to 1.5-fold in CD4⁺ T cells (Fig. 2, E and F). Because IL-2–driven Myc induction depends on STAT5 (2), this early Myc increase in CD8⁺, but not in CD4⁺, T cells likely reflects the sustained pSTAT5 seen in CD8⁺ T cells.

IL-2–dependent proliferation in T cell blasts also requires activation of the mechanistic target of rapamycin (mTOR) signaling, but not ERK-MAPK signaling (5). As a proxy for mTOR activity, we monitored phosphorylated ribosomal protein S6 (pS6) over time in CD4⁺ and CD8⁺ T cell blasts. Whereas CD4⁺ T cells required \sim 2 hours before most of the cells had activated pS6, almost all CD8⁺ T cells activated S6 immediately after stimulation. The mean fluorescence intensity of pS6 also increased throughout the time course for CD8⁺ T cells (fig. S2, A and B). Because maximal, sustained pS6 depends on STAT5 activation (23, 24), this delayed, weaker pS6 activation in CD4⁺ cells likely reflects the early attenuation of pSTAT5.

Sustained signaling requires protein synthesis in CD4⁺, but not in CD8⁺, T cells

Could these distinct signaling dynamics explain the difference in proliferative responses to IL-2? To address this question, we first needed to understand the mechanistic basis for these patterns. Washout experiments suggested that both the second wave of pSTAT5 in CD4⁺ and sustained signaling in CD8⁺ T cells required the continued presence of IL-2, perhaps to initiate new signaling complexes between IL-2 and unoccupied receptors (Fig. 3, A and B). After adding a small-molecule inhibitor of JAK3 to terminate new signal generation, pSTAT5 decayed rapidly in both cell types, indicating that STAT5 is dephosphorylated at similar rates in CD4⁺ and CD8⁺ T cells (Fig. 3, C and D).

The washout and inhibitor studies led us to suspect that the attenuation of pSTAT5 in CD4⁺ T cells was due to a relative loss of active

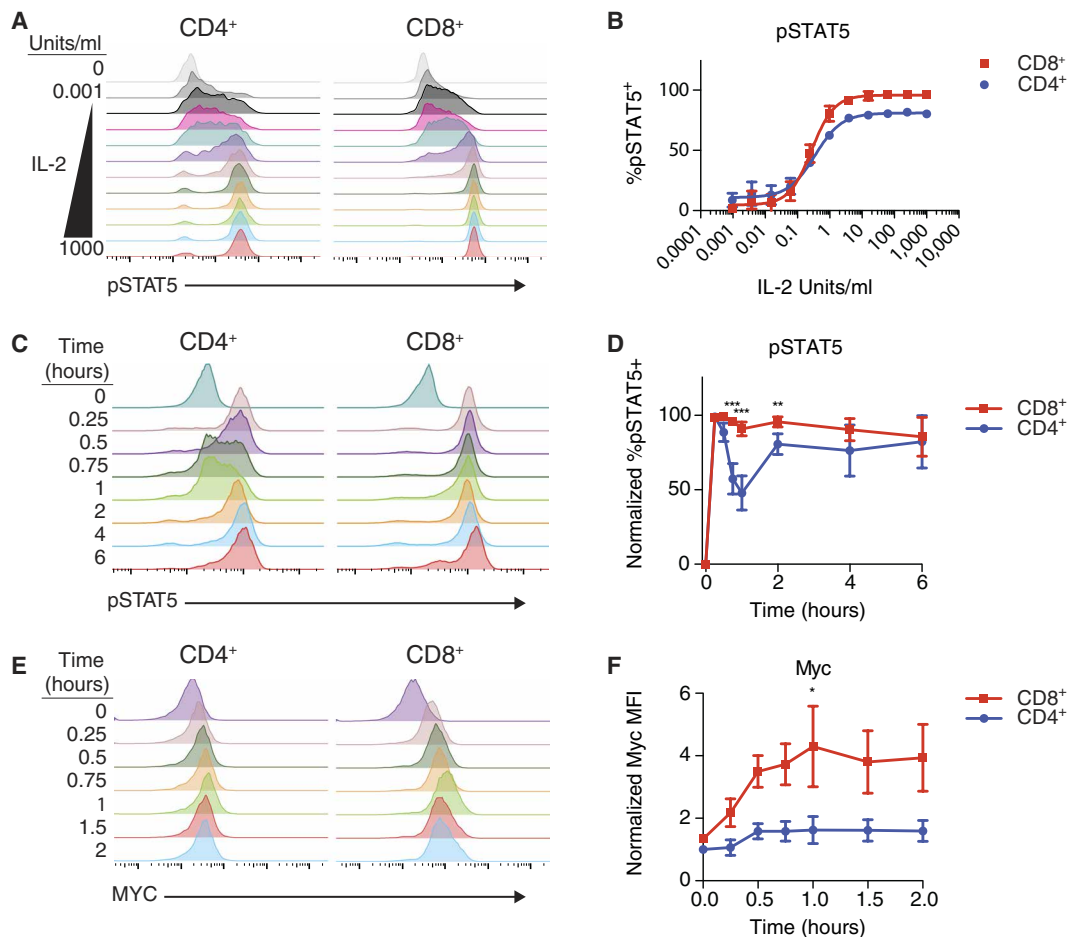


Fig. 2. Distinct IL-2 signaling dynamics in CD4 and CD8 T cells. (A) Rested CD4⁺ and CD8⁺ T cell blasts were stimulated with a fourfold titration of IL-2 from 1000 to 0.001 U/ml, and phosphorylated signal transducer and activator of transcription 5 (pSTAT5) was assessed by phosphoflow cytometry. (B) Fraction of responding (pSTAT5⁺) cells at each concentration of IL-2. EC₅₀ (median effective concentration) CD4 0.35 U/ml [95% confidence interval (CI), 0.29 to 0.42]; EC₅₀ CD8 0.28 U/ml (95% CI, 0.24 to 0.32). (C) Time course of pSTAT5 accumulation in CD4⁺ and CD8⁺ T cells after IL-2 stimulation. (D) Quantification of pSTAT5 over time (cell type, time, and interaction, all $P < 0.0001$). (E) Myc protein accumulation in CD4⁺ and CD8⁺ T cells. (F) Quantification of Myc over the first 2 hours of IL-2 stimulation [cell type, $P < 0.0001$; time, $P = 0.045$; interaction, not significant (n.s.)]. Representative histograms from 3 (A), 11 (C), or 4 (E) independent experiments. Plotted as mean \pm SEM of 3 (B) or 4 (F) independent experiments or mean with 95% CI from 11 independent experiments (D). Time courses compared by repeated-measures ANOVA with Bonferroni multiple comparison test (significant $P < 0.05$, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ for CD4s versus CD8s at a given time point).

signaling complexes rather than a differential phosphatase activity. At a minimum, STAT5 phosphorylation requires the IL-2R β and IL-2R γ chains and the cytoplasmic kinases JAK1 and JAK3. Upon IL-2 binding, the IL-2 receptor complex is rapidly internalized, and IL-2R β and IL-2R γ are degraded (13, 14). This creates a shortage of receptors to generate new pSTAT5, absent trafficking, and possibly synthesis of new receptors. Both cell types required vesicular transport to sustain signaling, because cotreatment with IL-2 and the Arf-guanine nucleotide exchange factor inhibitor brefeldin A gave a transient pSTAT5 peak that rapidly decayed after 15 min (Fig. 3, E and F). To distinguish between vesicular transport of newly synthesized signaling components and a presynthesized intracellular pool, we treated cells with both IL-2 and the protein synthesis inhibitor cycloheximide. Strikingly, pSTAT5 was completely unaffected by cycloheximide in CD8⁺ T cells. By contrast, pSTAT5 decayed in CD4⁺ T cells after the initial peak, indicating that protein synthesis was required for the second wave of signaling in CD4⁺ T cells (Fig. 3, G and H). These results suggested that CD8⁺ T cells had sufficient quantities of extant signaling components in an intracellular

pool to sustain STAT5 phosphorylation, whereas CD4⁺ T cells needed to resynthesize a limiting component or components and transport it to the cell surface for the second wave of STAT5 phosphorylation to occur.

IL-2R β and IL-2R γ are twice as abundant in CD8⁺ T cells as in CD4⁺ T cells

Given the known short half-life of signaling-competent receptors and the distinct requirement for new protein synthesis, we suspected that differences in initial receptor abundance could underlie the distinct signaling dynamics in CD4⁺ and CD8⁺ T cells. Surface staining of IL-2 receptor chains revealed that CD8⁺ T cells have about twice as much IL-2R β and IL-2R γ as CD4⁺ T cells, with no significant difference in IL-2R α abundance (Fig. 4, A and B). To probe the intracellular pool specifically, we bound surface receptors with unlabeled antibodies before fixation and membrane permeabilization and then stained for each receptor with the same fluorophore-antibody conjugate as used in the surface-only experiments. Using this method, we observed that intracellular IL-2R α staining was much weaker than

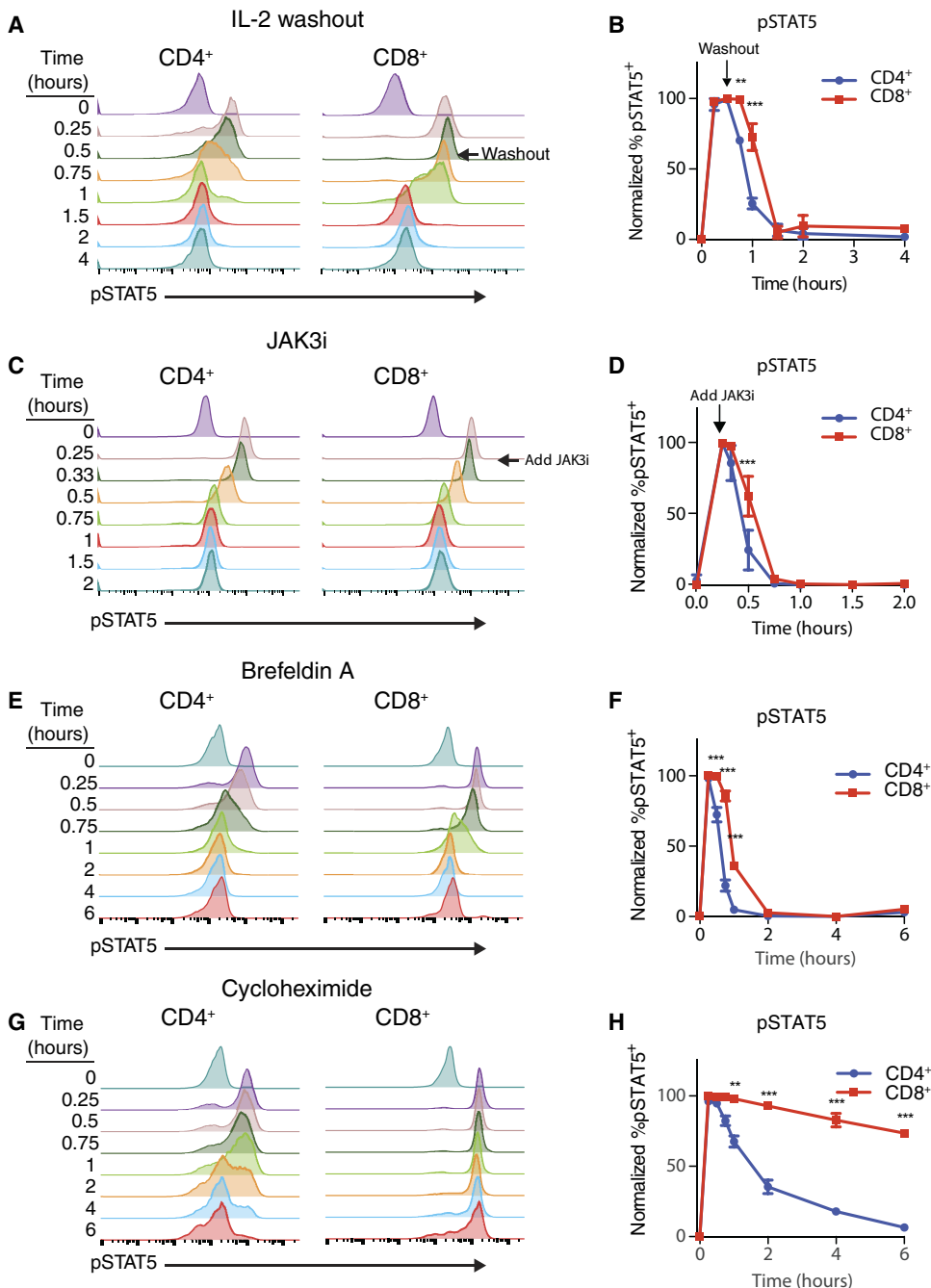


Fig. 3. Requirements for sustained IL-2 signaling. (A) Cells were stimulated for 30 min, and then IL-2 was washed out and pSTAT5 was monitored by phosphoflow cytometry. (B) Quantification of washout. (C and D) Cells were stimulated with IL-2, with a pharmacological inhibitor of JAK3 (JAK3i) added at $t = 15$ min, and signal decay was monitored (C) and quantified (D). (E and F) Cells were stimulated simultaneously with IL-2 and brefeldin A as pSTAT5 was monitored over time (E) and quantified (F). Cell type, time, and interaction, all $P < 0.0001$. (G and H) Cells were stimulated simultaneously with IL-2 and cycloheximide as pSTAT5 was monitored over time (G) and quantified (H). In all experiments, cell type, time, and interaction, all $P < 0.0001$. Data are representative of three independent experiments (A, C, E, and G) or plotted as means \pm SEM of three independent experiments (B, D, F, and H). Time courses compared by repeated-measures ANOVA with Bonferroni multiple comparison test (significant $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ for CD4s versus CD8s at a given time point).

surface staining, suggesting a predominantly surface localization, and was not significantly different between CD4⁺ and CD8⁺ T cells (Fig. 4, C and D). In contrast to IL-2R α , there was strong intracellular staining

the dynamics of STAT5 phosphorylation can be tuned by IL-2R β abundance. A prediction of this model is that reducing IL-2R β in CD8⁺ T cells would result in a CD4⁺ T cell-like signaling pattern. Because

for IL-2R β and IL-2R γ , which indicated substantial intracellular pools, and these pools were significantly larger in CD8⁺ T cells by about 2- and 1.5-fold, respectively (Fig. 4, C and D). For IL-2R β , this difference appeared to be encoded at the mRNA level. Differences in transcript abundance did not correlate with protein for either IL-2R α or IL-2R γ , which suggests that there may be important differences in posttranscriptional regulation of these subunits between CD4⁺ and CD8⁺ T cells (Fig. 4E).

IL-2R β -dependent IL-15 signaling is biphasic in CD4⁺, but not in CD8⁺ T cells

The increased IL-2R β and IL-2R γ on CD8⁺ T cells suggested that differences in abundance of either or both receptor chains could explain the distinct signaling dynamics observed in response to IL-2. Within the common γ chain family of cytokines (γ_c), two cytokines (IL-2 and IL-15) use the IL-2R β chain, with other members of this family signaling through a unique chain that pairs with IL-2R γ (Fig. 5A). To test the respective roles of IL-2R β and IL-2R γ , we compared signaling dynamics in response to IL-7 (which signals through IL-7R α and IL-2R γ) and IL-15 (which signals through IL-2R β and IL-2R γ). If IL-2R γ were limiting, then IL-2, IL-7 and IL-15 would all exhibit biphasic signaling, whereas limiting amounts of IL-2R β would only affect IL-2 and IL-15. After stimulation of CD4⁺ T cells with IL-7, pSTAT5 was strong and sustained, with no loss of signal until 5 hours after IL-2 addition (Fig. 5, B and C). In marked contrast, stimulation with IL-15 produced a biphasic response, with a significant loss of signal at 1 hour and subsequent partial recovery (Fig. 5, B and C). When CD8⁺ T cells were stimulated with either cytokine, pSTAT5 was strong and sustained (Fig. 5, D and E). Together, these results are consistent with a limiting pool of IL-2R β in CD4⁺ T cells underlying the rapid decline of pSTAT5 after stimulation with IL-2 or IL-15.

Reduction of IL-2R β abundance converts signaling in CD8⁺ T cells to a CD4⁺-like pattern

These results suggested a model in which the dynamics of STAT5 phosphorylation can be tuned by IL-2R β abundance. A prediction of this model is that reducing IL-2R β in CD8⁺ T cells would result in a CD4⁺ T cell-like signaling pattern. Because

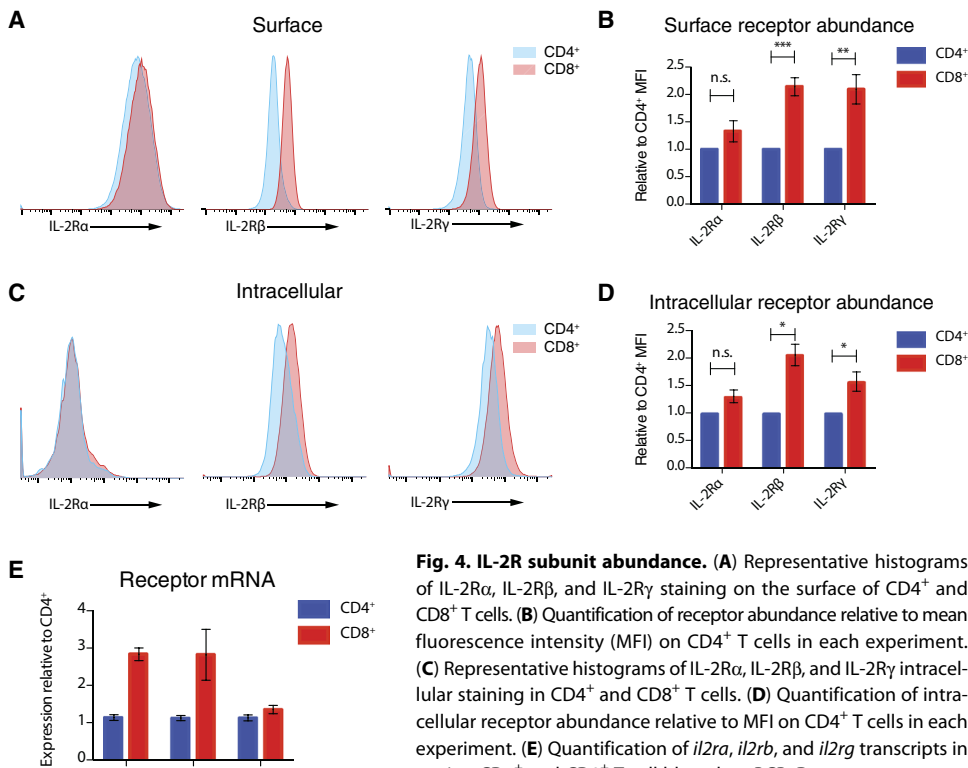


Fig. 4. IL-2R subunit abundance. (A) Representative histograms of IL-2R α , IL-2R β , and IL-2R γ staining on the surface of CD4 $^{+}$ and CD8 $^{+}$ T cells. (B) Quantification of receptor abundance relative to mean fluorescence intensity (MFI) on CD4 $^{+}$ T cells in each experiment. (C) Representative histograms of IL-2R α , IL-2R β , and IL-2R γ intracellular staining in CD4 $^{+}$ and CD8 $^{+}$ T cells. (D) Quantification of intracellular receptor abundance relative to MFI on CD4 $^{+}$ T cells in each experiment. (E) Quantification of *il2ra*, *il2rb*, and *il2rg* transcripts in resting CD4 $^{+}$ and CD8 $^{+}$ T cell blasts by qPCR. Data are representative of seven (A) and three (C) independent experiments. Pooled

data from seven (B) and three (D) independent experiments, plotted \pm SEM. Paired *t* test: **P* < 0.05, ****P* < 0.01, *****P* < 0.001. (E) mRNA from three biological replicates plotted with 95% CI.

il2rb $^{-/-}$ T cells have roughly similar IL-2R β abundance to wild-type T cells (24), we transduced primary CD8 $^{+}$ T cell blasts with lentiviruses expressing green fluorescent protein (GFP) and one of a panel of four small hairpin RNAs (shRNAs) targeting *il2rb*. These hairpins gave a range of knockdown efficiencies from 22 to 77% based on surface staining (Fig. 6A and fig. S3A). Reduction of IL-2R β in CD8 $^{+}$ T cells by \sim 50% with IL2RB shRNA #2 or #3 resulted in a biphasic CD4 $^{+}$ -like signaling pattern, with a loss of pSTAT5 at 1 hour, followed by rapid recovery (Fig. 6, B and C, and fig. S3B). Scrambled shRNA or a marginal reduction with IL2RB shRNA #4 had a minimal effect on signaling dynamics (Fig. 6, B and C, and fig. S3B). Knockdown to below 25% of wild-type abundance with IL2RB shRNA #1 markedly impaired signaling, producing a very weak pSTAT5 response at all time points (Fig. 6C and fig. S3B). Twofold reduction in IL-2R β with shRNA #2 had only a small effect on the maximal pSTAT5 response observed at 15 min (fig. S3C), which was comparable to the difference observed between CD4 $^{+}$ and CD8 $^{+}$ T cells (Fig. 2D).

We next asked whether this reduction in IL-2R β abundance contributed to the weaker proliferative response of CD4 $^{+}$ T cells. Sustaining primary CD8 $^{+}$ T cells in culture was challenging in the context of impaired IL-2 signaling. After culture for 3 days in the presence of IL-2, the frequency of cells expressing IL2RB shRNAs #2 and #3 had decreased by six- and threefold, respectively, but there was no substantial change in the frequency of scrambled shRNA-expressing cells (fig. S3D). This result could be explained by defects in either proliferative or survival signaling, so we next assessed S phase entry in cells that were activated for 3 days, rested overnight, and then stimulated with IL-2 for 13 hours. Under these conditions, a significantly smaller fraction of

IL2RB shRNA-expressing cells entered the S phase compared to untransduced cells in the same sample (Fig. 6D). Although additional mechanisms likely contribute, our results indicate that even a modest two-fold difference in IL-2R β abundance is sufficient to confer a proliferative advantage on CD8 $^{+}$ T cells relative to CD4 $^{+}$ T cells in response to IL-2 stimulation.

T_{regs} have increased IL-2R β abundance and CD8 $^{+}$ -like signaling dynamics

To test whether IL-2R β abundance was sufficient to tune signaling dynamics, we attempted to overexpress IL-2R β in CD4 $^{+}$ T cell blasts. Unfortunately, this proved technically challenging, so we instead assessed IL-2R β abundance in other CD4 $^{+}$ T cell subsets to see if any had a CD8 $^{+}$ -like IL-2R β pool. Intracellular IL-2R β was about two-fold more abundant in freshly isolated splenic T_{regs} than in effector CD4 $^{+}$ T cell blasts (Fig. 7, A and B). This intracellular pool was similar to that found in CD8 $^{+}$ T cell blasts, which our model predicts is sufficient to support a strong and sustained IL-2 pSTAT5 response. Upon IL-2 stimulation, we observed a rapid increase in pSTAT5 that was sustained throughout a 4-hour time course (Fig. 7,

C and D). Although only correlative, these results provide further evidence of a crucial role for IL-2R β abundance in tuning the dynamics of the IL-2 pSTAT5 response.

DISCUSSION

Research in multiple systems has recognized the important role that signaling dynamics play in quantitatively altering cell responses (25), as in the case of epidermal growth factor and tumor necrosis factor α signaling (26, 27). In these previous studies, the authors altered signaling dynamics by modulating the stimulus. Here, we observe different signaling dynamics in two closely related cell types in response to the same stimulus. A 50% lower abundance of one component of the IL-2 receptor complex, IL-2R β , appears to explain the altered dynamics and contribute to the decreased proliferative response of CD4 $^{+}$ T cells to IL-2. In the context of receptor assembly, this is the logical step at which to specifically regulate the pro-proliferative and growth signals of IL-2 and IL-15 without affecting other γ -cytokines. Although there is debate as to whether IL-2 first binds a preformed IL-2R α /IL-2R β heterodimer (28, 29) or first to IL-2R α and then IL-2R β (6, 30) before IL-2R γ is recruited, a shortage of IL-2R β would disfavor the formation of the final quaternary IL-2/IL-2R α /IL-2R β /IL-2R γ complex required for signaling in either model (29). On CD4 $^{+}$ T cell blasts, there appears to be sufficient IL-2R β to maximally phosphorylate STAT5 at early time points after IL-2 stimulation. However, our data suggest that between 15 min and 1 hour, the number of competent signaling complexes falls below the threshold needed to counteract phosphatase activity and maintain STAT5 phosphorylation. Synthesis of new IL-2R β

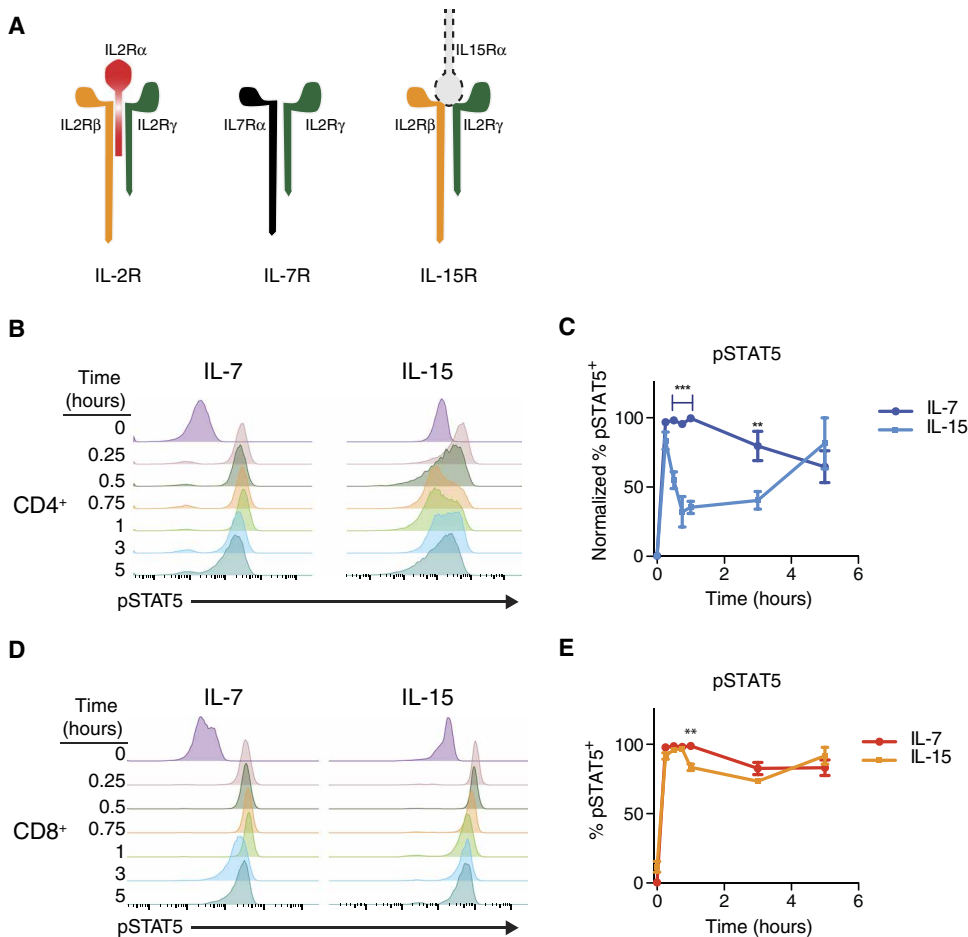


Fig. 5. Comparative dynamics of IL-7 and IL-15. (A) Schematic of IL-2, IL-7, and IL-15 receptor components. Note that IL-15 is typically presented in trans from IL-15R α on a neighboring cell. (B) Representative time course histograms of pSTAT5 in CD4⁺ T cells stimulated with IL-7 or IL-15. (C) Fraction of pSTAT5⁺ cells in response to IL-7 and IL-15. Cytokine, time, and interaction, all $P < 0.0001$. (D) Representative time course histograms of pSTAT5 in CD8⁺ T cells stimulated with IL-7 or IL-15. (E) Quantification of pSTAT5 in response to IL-7 or IL-15. Cytokine, n.s.; time, $P < 0.0001$; interaction, $P = 0.0004$. Data are representative of (B and D) or pooled from (C and E) three independent experiments. Plotted \pm SEM in (C) and (E). Time courses compared by repeated-measures ANOVA with Bonferroni multiple comparison test (significant $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ for IL-7 versus IL-15 at a given time point).

molecules enables the second wave of STAT5 phosphorylation in CD4⁺ T cells. By contrast, the total pool of IL-2R β on CD8⁺ T cells is large enough to maintain surface receptor abundance above this threshold even in the absence of new protein synthesis and, thus, sustain STAT5 phosphorylation continuously.

Although CD4⁺ T cells do ultimately have a second, sustained wave of STAT5 phosphorylation, the early loss of pSTAT5 occurs at a crucial time for induction of immediate-early genes. In fibroblasts, small differences in the duration of ERK activity (90 min versus 120 min) profoundly affect the induction of Myc and the time to cell cycle progression (31). Here, we observe higher Myc abundance in CD8⁺ T cells after just 2 hours. This likely has a cascading effect on many genes required for cell cycle entry and contributes to the hyperproliferative response.

Why are these two cell types programmed to respond to IL-2 with distinct signaling dynamics? We speculate that this may serve as an additional check against the unintentional or mislocalized expansion

of CD4⁺ T cells in response to IL-2. Because IL-2 has a very short effective range in vivo ($<20 \mu\text{m}$) (32, 33), only a T cell that maintains close proximity with an IL-2-producing source (such as a peptide-APC-bound T cell) would receive sufficient exposure for a first and second wave of STAT5 phosphorylation. This would prevent the expansion of a CD4⁺ T cell that once saw antigen (perhaps self-antigen) and by chance circulated through an area of active infection. CD4⁺ T cells respond to infections by recruiting and activating other immune cells, many of which are nonspecific and have the potential to cause harmful inflammation (34). This mechanism both necessitates tight control over CD4⁺ expansion to prevent autoimmunity and minimizes the need for massive expansion because other cells will amplify the response. By contrast, CD8⁺ T cells primarily respond by directly killing virally infected cells with lytic granules (35). Unlike the nonspecific effectors downstream of CD4⁺ T cells, CD8⁺ T cell-mediated killing requires recognition of the correct peptide-bound major histocompatibility complex on target cells, which mitigates the need for tightly regulated IL-2 responses. This direct killing mechanism necessitates the opposite: A rapid and large expansion of CD8⁺ T cells is required because there is little amplification by other effector cells.

To focus on the consequences of IL-2 signaling specifically, we studied preactivated T cell blasts that were no longer receiving TCR stimulation. Recent work from our laboratory suggests that IL-2R β abundance correlates with responses in the context of simultaneous TCR and IL-2 signals (11). T cells integrate both of these signals at the level of Myc when deciding whether or how much to proliferate (11, 22). However, IL-2 augments TCR-driven Myc induction only in CD8⁺ T cells (11). This enables CD8⁺, but not CD4⁺, T cells to proliferate in response to suboptimal TCR stimulation. Even 24 hours after TCR activation, CD4⁺ T cells have a very minimal STAT5 phosphorylation response to IL-2 (similar to 75% knockdown of IL-2R β in Fig. 5C). These activated CD4⁺ T cells express very little IL-2R β and may represent a physiologic example of the IL-2R β tuning described here.

Our data and previous reports show that there is significant heterogeneity in IL-2R β abundance, both between and within cell populations, which has an important impact on signaling and cell fate decisions. At the low end, naïve CD4⁺ T cells have little or no IL-2R β , whereas at the high end, natural killer (NK) cells have enormous quantities (36). Here, we showed that for T_{regs}, as well as CD4⁺ and CD8⁺ effectors, mean IL-2R β abundance at the population level tunes IL-2 signaling dynamics. Recently, Jansen *et al.* examined IL-2 signaling dynamics in human NK cells and found, as our model would predict, that these also had a sustained

Fig. 6. Knockdown of IL2RB in CD8 T cell blasts.

(A) Effect of expressing four different shRNAs targeting IL2RB relative to scrambled shRNA on surface IL-2R β abundance in CD8⁺ T cells, as assessed by fluorescence-activated cell sorting (FACS). (B) pSTAT5 over time in IL-2–treated CD8⁺ T cells expressing the scrambled IL2RB shRNA or IL2RB shRNA #2. (C) Fraction of pSTAT5⁺ cells over time. [Repeated-measures ANOVA: shRNA, $P < 0.0001$; time, $P < 0.0001$; interaction, $P = 0.0097$. Bonferroni posttest of scramble shRNA versus shRNA #1: 0.25 to 3 hours ($P < 0.001$); versus shRNA #2: 0.75 and 1 hour ($P < 0.001$); versus shRNA #3: 0.75 hour ($P < 0.05$), 1 hour ($P < 0.001$); all other comparisons to scramble (n.s.).] (D) S phase entry, assessed by EdU incorporation, after 13 hours of IL-2 stimulation in briefly rested CD8⁺ T cell blasts expressing the indicated shRNAs (GFP⁺) or untransduced (GFP⁻) cells. *** $P < 0.001$ by one-way ANOVA with Bonferroni multiple comparison test relative to scramble shRNA. Data are representative of (B) or pooled from four (A) or three (B to D) independent experiments. Plotted as means \pm SEM.

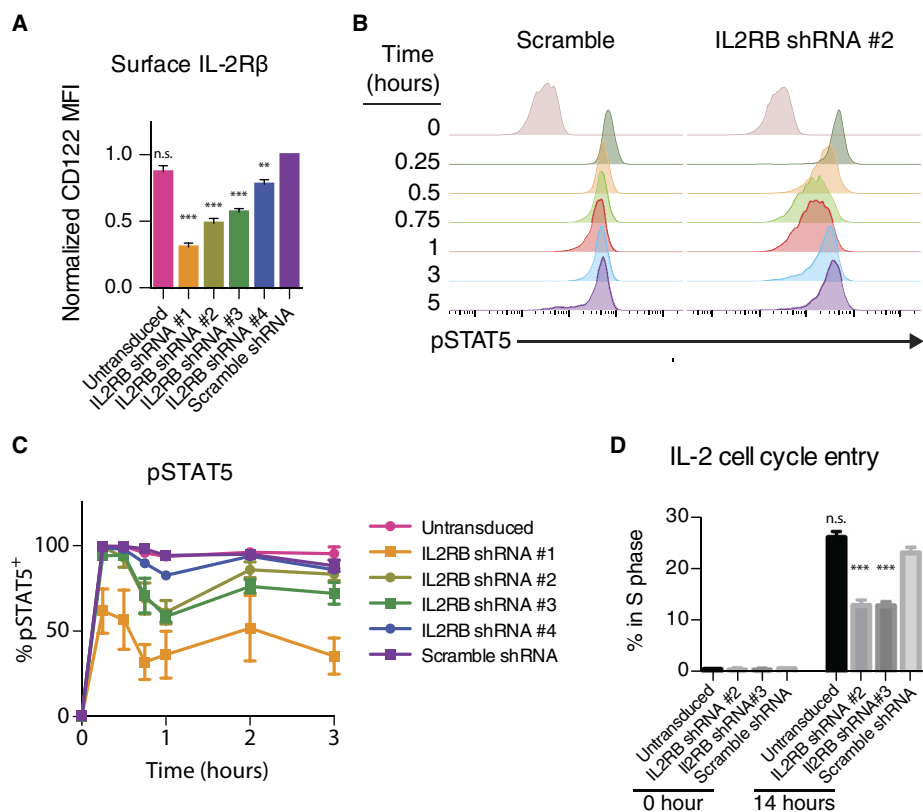
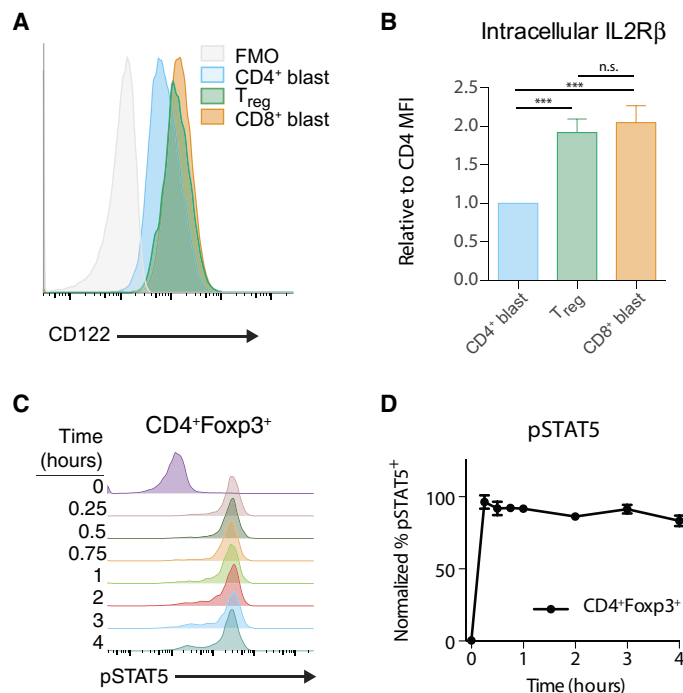


Fig. 7. T_{reg} IL-2R β abundance and IL-2 signaling dynamics.

(A) Representative intracellular staining for IL-2R β in CD4⁺ T cell blasts, CD4⁺Foxp3⁺T_{reg}s, and CD8⁺ T cell blasts. (B) Quantification of IL-2R β abundance relative to CD4⁺ T cell blasts. (C) Representative histograms of STAT5 phosphorylation over time after IL-2 stimulation of CD4⁺Foxp3⁺ T_{reg}s in freshly isolated splenocytes, quantified in (D). All graphs plotted as mean \pm SEM. In (B), T_{reg}s are from four separate mice, and blasts are from four separate preparations. *** $P < 0.001$ by one-way ANOVA with Bonferroni multiple comparison test. In (D), data are pooled from five mice.



response over a 24-hour period (36). Future work should explore this relationship in other cell types and, when reagents permit, correlate IL-2R β abundance to the STAT5 phosphorylation response within specific populations to further understand this heterogeneity.

Cell fate decisions in response to cytokines and other stimuli require sustained signaling. Our results highlight how a focus on immediate signaling can be deceptive when attempting to understand events that occur hours later. Here, a mere twofold difference in receptor abundance

yields distinct dynamics that contribute to quantitatively different proliferative outcomes.

MATERIALS AND METHODS

Mice

The C57BL/6J mice (Jackson) used in this study were housed in specific pathogen-free facilities at the University of California, San Francisco (UCSF), and were treated according to protocols approved by the Institutional Animal Care and Use Committee in accordance with U.S. National Institutes of Health (NIH) guidelines. Both male and female mice, all aged 6 to 12 weeks, were used as a source of primary T cells.

Reagents

The JAK3-selective inhibitor (JAK3i) was synthesized as previously described (5) and used at a concentration of 500 nM. Recombinant human IL-2 (rhIL-2) was from the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH; Maurice Gately (Hoffmann-La Roche). Recombinant murine IL-7 and IL-15 were purchased from PeproTech.

Primary T cell culture

T cells were purified from single-cell suspensions of spleen and lymph nodes from male and female mice aged 6 to 12 weeks by negative selection with biotinylated antibodies recognizing CD8 or CD4, CD19, B220, CD11b, CD11c, DX5, Ter119, and CD24 (UCSF Monoclonal Antibody Core) and magnetic anti-Biotin beads (MACSi Beads, Miltenyi Biotec). For IL-2 stimulation, purified T cells were preactivated on six-well plates coated with antibodies specific for CD3 (2C11) and CD28 (37.51) for 72 hours, removed, and cultured with rhIL-2 (100 U/ml, Roche) for 36 hours, and then cultured without rhIL-2 for the 36 hours before all experiments.

IL-2 stimulation of T cells

After resting for 36 hours without IL-2, T cells were washed in fresh media, live/dead-stained as appropriate (see Flow Cytometry), and resuspended in warm, fresh RPMI complete media at 1×10^6 /ml to 5×10^6 /ml. Cells were then stimulated by adding rhIL-2 (20 \times stock in RPMI complete, 50 U/ml final concentration) and assayed as detailed below. To wash out IL-2 after stimulation, we washed the cells three times with 1 ml of warmed media and resuspended them at the original concentration.

T cell proliferation

Preactivated T cells were cultured in 24-well plates for 10 to 30 hours with IL-2 and then pulsed with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU) for 1 hour and assayed per the manufacturer's procedure (Click-IT Plus EdU, Life Technologies). Alternatively, cells were loaded with CellTrace Violet (Life Technologies), plated into 96-well plates, and stimulated with IL-2 for 72 hours. To maintain viability in the undivided population, we treated control (non-IL-2-stimulated) cells with low-dose IL-7 (0.1 ng/ml). Division was then analyzed by quantifying EdU incorporation by flow cytometry.

Flow cytometry

Cells were live/dead-stained using the Live/Dead Fixable Near IR Dead Cell Stain Kit or Live/Dead Fixable Violet Dead Cell Stain Kit (Life Technologies). For surface stains, cells were stained for 30 min on ice with the indicated antibodies. Information on antibody clones, sources, and working dilutions are listed in table S1. For intracellular stains, sam-

ples were fixed at the indicated time after stimulation in 2% paraformaldehyde, surface-stained with CD8a-BUV737 and CD4-BUV395, fixed again, and then permeabilized with ice-cold 90% methanol at -20°C overnight. Samples were then barcoded using Pacific Orange-NHS ester (0.33 or 5 μ g/ml), Pacific Blue-NHS Ester (0.67 or 10 μ g/ml), and AlexaFluor (AF) 488-NHS Ester (0.26 or 2 μ g/ml) (Life Technologies), as previously described (5). Intracellular antigens were then stained for 30 min at 23°C with antibodies indicated in table S1. Samples were acquired on a BD LSR Fortessa SORP and analyzed in FlowJo (Tree Star).

Quantitative polymerase chain reaction

RNA was isolated from 1×10^6 to 2×10^6 T cell blasts per condition using RNeasy kit (Qiagen), and cDNA was synthesized using qScript (Quanta Biosciences). mRNA was detected by PrimeTime (IDT) or TaqMan (Life Technologies) predesigned quantitative polymerase chain reaction (qPCR) assays. Primer and probe sequences can be found in table S2. Data are from three replicates collected on a QuantStudio 12k (Life Technologies), plotted with 95% confidence intervals as calculated by Quantstudio (Life Technologies).

IL2RB knockdown

Four shRNAs targeting IL2RB and one scrambled shRNA were purchased (pLKO1.0, The RNAi Consortium/Sigma-Aldrich). Sequences of the shRNAs are provided in table S3. The puromycin selection cassette was replaced with enhanced GFP by restriction enzyme cloning. Lentivirus was produced in human embryonic kidney-293T cells in 10-cm dishes transfected with pCMV-dr8.91 (9 μ g), pMD2.G (0.9 μ g), and pLKO (9 μ g) in the presence of Viral Boost (Alstem). CD8⁺ T cells were isolated as described above and incubated for 1 day on TCR-coated plates before spin transduction (1 hour, 2200 rpm, 23°C) with viral supernatant in the presence of IL-2 (100 U/ml). Cells were left on TCR plates through day 3 and treated as per culture conditions described above. Assays were conducted as described above except for an additional anti-GFP-AF488 stain, which was used to detect GFP in the EdU incorporation assay for S phase entry.

Statistical analysis

Data were analyzed in Prism (GraphPad). Three types of statistical analysis were used: (i) Repeated-measures analysis of variance (ANOVA) with Bonferroni multiple comparison posttest was used to compare signaling time courses between cell types, stimulatory cytokines, or shRNA transformations. (ii) Paired *t* test was used to compare IL-2R subunit levels between CD4 and CD8 T cells (paired samples were stained and collected together on the same day in the same tube). (iii) One-way ANOVA with Bonferroni multiple comparison posttest was used to compare IL-2Rb levels between different shRNA constructs and to compare S phase entry between different shRNA constructs.

SUPPLEMENTARY MATERIALS

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Fig. S1. STAT5 phosphorylation in IL-2-stimulated CD4⁺ and CD8⁺ T cells.

Fig. S2. PI3K-mTOR signaling in IL-2-stimulated CD4⁺ and CD8⁺ T cells.

Fig. S3. IL2RB shRNAs.

Table S1. FACS antibodies.

Table S2. qPCR assays.

Table S3. IL2RB shRNAs.

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IL-2R β abundance differentially tunes IL-2 signaling dynamics in CD4 $^+$ and CD8 $^+$ T cells

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Receptor abundance defines signaling dynamics

Although the cytokine interleukin-2 (IL-2) stimulates the same receptors and intracellular signaling components in CD4 $^+$ and CD8 $^+$ T cells, these cells exhibit different responses to IL-2. Both cell types proliferate in response to IL-2, but CD8 $^+$ T cells enter the S phase earlier and are more proliferative than CD4 $^+$ T cells. Smith *et al.* found that whereas IL-2 stimulated sustained phosphorylation of the transcription factor STAT5 in CD8 $^+$ T cells, it caused CD4 $^+$ T cells to exhibit two phases of STAT5 phosphorylation. The IL-2 receptor subunit IL-2R β was less abundant in CD4 $^+$ T cells than in CD8 $^+$ T cells. Reducing IL-2R β in CD8 $^+$ T cells made these cells exhibit a CD4 $^+$ T cell –like pattern of STAT5 phosphorylation and delayed cell cycle entry. These distinct IL-2 signaling dynamics may tune the responses of CD4 $^+$ and CD8 $^+$ T cells to enable an adequate immune response while protecting against autoimmunity.

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