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Dissecting common γ chain cytokine family signaling in T cells using cell-to-cell variability analysis

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Abstract

Natural variability in abundance of signaling regulators can lead to divergence in cell fate, even within genetically identical cells sharing a common differentiation state. To leverage this observation, we introduce cell-to-cell variability analysis (CCVA), an experimental and computational methodology to quantify the correlation between variability in signaling regulator abundance and variation in sensitivity to stimuli. Here, we apply CCVA to investigate the unexpected effects of the interleukin 2 (IL-2) receptor α chain (IL-2R α) on the sensitivity to common- γ chain (γ_c) cytokines in primary T lymphocytes. Our work demonstrates that increased IL-2R α abundance decreases the concentration of IL-2 but increases the concentrations of IL-7 and IL-15 required for a half-maximal activation (EC₅₀) of downstream signal transducer and activator of transcription 5 (STAT5), without affecting the EC₅₀ of other γ_c cytokines. To probe the mechanism of IL-2R α 's effect on γ_c family cytokine EC₅₀s, we introduce a Bayesian-inference computational framework that models the formation of receptor signaling complexes using prior biophysical measurements. Using this framework, we demonstrate that a model in which IL-2R α drives γ_c depletion through pre-assembly of complete IL-2 receptors is consistent with both CCVA data and prior measurements. The combination of CCVA and computational modeling yields quantitative understanding of the crosstalk of γ_c cytokine signaling in T lymphocytes.

INTRODUCTION

Quantifying the impact of protein abundance on cellular function has attracted considerable attention in recent years (1–4). To do so in bacteria, researchers have changed incrementally the abundance of a chosen protein and measure the functional consequences (5–9).

However, this approach is more cumbersome in primary mammalian cells, such that protein function has been principally studied in an all-or-nothing fashion using genetic mutants or RNAi. As an alternative, we propose that inherent natural variability in protein abundance, as recently observed within populations of genetically identical mammalian cells (10–15), can be used to dissect the quantitative regulation of signal transduction.

To assess the phenotypic variability of populations of isogenic cells, researchers can quantify the variability abundance of mRNA or protein with individual cell resolution using

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single cell RT-qPCR (16) or flow cytometry (12, 13). Of note, studies utilizing these techniques have demonstrated large heterogeneity in the abundance of signaling components (receptors, kinases, adapters, phosphatases and cytokines), with typical coefficients of variation (CV) for the lognormal distribution of mRNA and protein amounts larger than 0.5 within activated T cell clones for example (16, 17). Concretely, in such distributions, 15% of cells will have protein abundances deviating from the median by more than two fold. Even larger variability was uncovered in the case of the interleukin 2 (IL-2) receptor α chain (IL-2R α), with CVs of up to 3.0 in populations of genetically identical transgenic T cells activated in vitro (17). In these cells, 15% of the population has IL-2R α abundance that deviates from the median by more than 10 fold in either direction (17). In settings of infection, this variability in T cells' IL-2R α abundance has been shown to correlate with a split between short-lived effector or memory precursor fates (18). Examples in which a continuous spectrum of surface protein abundance maps onto discrete differentiation paths have been reported in other biological systems as well (12, 19). These observations raise the question of how variability in protein abundance affects signaling thresholds and ultimately cell differentiation decisions.

In this work, we present an experimental methodology to quantitatively correlate such variability in protein abundance with variable regulatory function. As part of this methodology, we present a software package, *ScatterSlice*, which quantitatively correlates the functional heterogeneity of cellular signaling thresholds with variation in protein abundance. We apply this methodology to probe the signaling responses to common γ_c chain (γ_c) cytokines, interleukins (IL-) 2, 4, 7, 9, 15 and 21, whose receptors share the γ_c chain (CD132) (20). IL-2 and IL-15 also share their β chain (CD122). Each cytokine receptor has an α chain that imparts specificity through enhanced affinity for its respective cytokine. For example, the IL-2 receptor is formed through the assembly of the γ_c and β chains with a specific α chain, IL-2R α (CD25) whereas the IL-7 receptor is composed of the γ_c and its specific α chain, IL-7R α (CD127). Though it has been established that expression of each cytokine's α chain is required for signaling (21), quantitative understanding of the effects of sharing a receptor chain on the ability of T cells to trigger downstream phosphorylation in response to γ_c cytokines remains elusive.

Specifically, we investigated whether variation in IL-2R α abundance influences sensitivity to IL-2 and other γ_c family cytokines. In agreement with previous results (17), we report that large variation in IL-2R α correlated with much of the variability in cells' sensitivity to IL-2. More surprisingly, we found that higher abundance in IL-2R α correlated with inhibited sensitivity to IL-7. Decreases in IL-7R α abundance at the cell surface or direct inhibition of IL-7R α signaling were ruled out as mechanisms explaining the dependency of IL-7 sensitivity on IL-2R α . Instead, a simple thermodynamic model suggested that large abundance of IL-2R α could inhibit IL-7 signaling indirectly, by sequestering γ_c chains into preformed heterotrimeric IL-2 receptors in the absence of IL-2. Extending our experimental system to other cytokines of the γ_c family, we determined that IL-15 sensitivity is slightly dampened by IL-2R α abundance, whereas sensitivities to IL-4 and IL-21 are unaffected.

RESULTS

Phenotypic variability between T cell populations leads to differences in IL-2 EC₅₀

We sampled isogenic populations of primary T cells at different timepoints following activation and found that a given concentration of IL-2 can trigger greatly varied signaling responses, as reflected in different degrees of STAT5 phosphorylation (Fig. 1A). We then analyzed T cells' pSTAT5 response to a complete dose-response of IL-2 after a 10-minute exposure. In one culture, we estimated the typical concentration (EC₅₀) of IL-2 that yields half-maximal pSTAT5 response to be 10 pM (Fig. 1B, green line); this is consistent with the

estimated dissociation constant (K_D) for the “high affinity IL-2 receptor” (22), as well as functional readouts such as cell proliferation of CTLL-2 cells (23). However, in other cultures, we observed shifts in IL-2 EC_{50} by ten-fold— up to 100 pM or down to 1 pM (Fig. 1B). Flow cytometry measurements of IL-2R α abundance revealed a close anti-correlation between IL-2 EC_{50} and the median abundance of IL-2R α (Fig. 1C). Cells from a culture with higher EC_{50} have lower median abundance of this receptor subunit, while cells from a culture with lower EC_{50} have greater median abundance. Overall, the median abundance of IL-2R α per cell correlates well with IL-2 EC_{50} ($R^2 = 0.87$; Fig. 1C).

Cell-to-cell variation in IL-2R α abundance leads to intra-population EC_{50} variation

Cells within a given population have differences in IL-2R α abundance of the same magnitude as differences in median IL-2R α abundance between populations (Fig. 1D). Since the variability in IL-2 EC_{50} between T cell cultures correlates well with variability in median IL-2R α abundance, we conjectured that the same correlation should exist when examining individual cells within a population.

This cell-by-cell assessment requires that IL-2R α abundance be measured with precision via flow cytometry. Dual labeling of IL-2R α with two different antibodies (Fig. 1E) demonstrates broad variation in either channel, but minimal variation in the ratio of two stains (Fig. 1F). Thus, even with the combined noise from staining with two antibodies and measurement across two channels, IL-2R α abundance can be determined with less than 1.5-fold error across a wide range (up to 10^5 -fold). Cell sorting experiments further confirm that distinct subpopulations can be accurately defined by their IL-2R α abundance (fig. S1).

To quantify systematically the correlation between cytokine EC_{50} and receptor abundance, we have developed a three-stage methodology, named cell-to-cell variability analysis (CCVA, Fig. 2A). First, cells are treated with a dose-response of stimulus. Second, protein abundance and downstream responses are measured concomitantly with single-cell resolution using flow cytometry. Finally, the flow cytometry data are analyzed with a software package named *ScatterSlice* (Fig. 2B–E). Conventional programs for flow cytometry analysis deliver snapshots of cellular response for a given stimulus dose, with the possibility of manually gating for subpopulations based on protein abundance. In contrast, our software was specially designed to automatically parse the heterogeneous population into subpopulations defined by protein abundance (Fig. 2B), quantify each population's downstream phosphorylation (Fig. 2C), then determine stimulus sensitivity using all doses of stimuli to fit an EC_{50} within each subpopulation (Fig. 2D). As a whole, CCVA delivers a complete map of the relationship between protein abundance and response sensitivity as quantified by the EC_{50} (Fig. 2E).

We applied CCVA to quantify the effect of variation in IL-2R α abundance on IL-2 EC_{50} within one population of T lymphocytes. We found that, as receptor abundance ranges from 10^2 to 10^7 , the EC_{50} of the IL-2 response decreases from 100 pM to 1 pM (Fig. 2E). Extending the same analysis to the different T cell populations shown in Figure 1 reveals that despite large differences in median IL-2R α abundance and corresponding shifts in the overall IL-2 EC_{50} from population to population (see Fig. 1C), an identical anti-correlation between IL-2R α abundance and EC_{50} is observed (fig. S2).

CCVA uncovers increased IL-7 EC_{50} correlating with increased IL-2R α abundance

In effector T cells, IL-2R α (i.e. CD25) is commonly used as a marker of activation, identifying an active effector phenotype between naïve and memory stages (24). Furthermore, effector T cells with lower abundance of IL-2R α have been shown to preferentially commit to a memory phenotype, whereas those with higher abundance of

IL-2R α do not (18). As both naïve and memory phenotypes are classically associated with enhanced sensitivity to IL-7 (25) we used CCVA to quantify the relationship between IL-2R α abundance and IL-7 EC₅₀. This analysis demonstrated a positive correlation between IL-2R α abundance and IL-7 EC₅₀, with increased IL-2R α abundance (from 10² to 10⁷ copies) scaling with increased EC₅₀ for IL-7 (from 3 to 60 pM) (Fig. 3A).

Decrease of IL-7 receptor abundance or signaling does not account for observed increase in IL-7 EC₅₀

The impact of IL-2R α abundance on IL-7 EC₅₀ could be hypothesized to stem from the reported temporal anti-correlation of IL-2R α and IL-7R α abundance during the course of differentiation from naïve to effector to memory phenotypes (25, 26). However, in our system, flow cytometric analysis demonstrated that abundances of IL-2R α and IL-7R α at the cell surface were not anti-correlated (fig. S3A). We confirmed this result by FACS sorting cells according to IL-2R α abundance and demonstrating by western blot a lack of correlation with IL-7R α abundance (fig. S3B). More critically, our investigations of population-level EC₅₀ revealed that although IL-7 EC₅₀ varies from 3 to 60 pM across populations, this variation does not correlate with IL-7R α abundance with any statistical significance (fig. S3C). This lack of correlation reflects a lack of uniformity in EC₅₀ despite fairly uniform abundance of IL-7R α across effector cell cultures, suggesting that factors other than IL-7R α abundance regulate IL-7 EC₅₀ in effector cells.

As an alternate mechanism, we tested whether IL-2R α abundance correlated with a direct inhibitory modification of the IL-7R α chain leading to fewer functional IL-7 receptors despite equal abundance. If this were the case, all cytokines signaling through IL-7R α would have reduced EC₅₀ correlating with higher abundance of IL-2R α . To test this possibility, we applied CCVA to characterize whether the EC₅₀ of T cells' response to thymic stromal lymphopoietin (TSLP)—a cytokine whose receptor also utilizes IL-7R α (fig. S4A)—also correlates with IL-2R α abundance. Our results showed no correlation between TSLP EC₅₀ and IL-2R α abundance (fig. S4B). Therefore, the increase in IL-7 EC₅₀ cannot be attributed to direct inhibition of IL-7R α signaling.

An equilibrium binding model of γ_c chain competition quantitatively accounts for IL-2R α effects on IL-7 EC₅₀

We then focused on the fact that IL-2 and IL-7 receptors share the γ_c chain (21, 27) (Fig. 3B). Specifically, we hypothesized that greater abundance of IL-2R α might drive the preformation of complete heterotrimeric IL-2 receptors composed of IL-2R α , IL-2R β , and γ_c chains. This sequestration of the γ_c chain in preformed IL-2 receptor complexes could lead to the increase in IL-7R α EC₅₀ with increased IL-2R α abundance, as reported in Fig. 3A.

Experimental evidence for existence of complete heterotrimeric IL-2 receptor complexes on the surface of living cells in the absence of cytokine remains equivocal. Indeed, the crystal structure of IL-2 in complex with the soluble extracellular portions of the full receptor indicates no contact between IL-2R α and either β or γ_c chains (28). Moreover, isolated extracellular domains of IL-2R α and γ_c have no detectable binding affinity in solution (29). On the other hand, the extracellular domains of IL-2R α and β have a binding affinity of 280 nM, as measured in solution (29). Furthermore, all pairwise interactions between IL-2R α , β and γ_c have been demonstrated on the surface of living cells in the absence of IL-2 (20, 30). It is therefore possible that in the absence of IL-2, high abundance of IL-2R α would drive the preformation of full heterotrimeric IL-2 receptor complexes.

In the following, we set out to investigate whether this hypothesis could realistically and quantitatively account for the correlation between IL-2R α abundance and IL-7 EC₅₀

observed in our CCVA experiments using an equilibrium binding model. We first compute the EC_{50} of the response to IL-2 and IL-7 from affinity constants (see Supplementary Material) and quantitatively compare these results to values measured by CCVA. We then identify the simplest equilibrium binding model with realistic affinity constants that can account for the correlation of IL-2 and IL-7 EC_{50} s with the abundance of IL-2R α uncovered by CCVA.

Estimating the cytokine EC_{50} using an equilibrium binding model for the receptor chains is appropriate because: 1) the system reaches a steady state within 10 minutes of exposure to cytokines (fig. S5); 2) pSTAT5 correlates linearly with receptor occupancy (fig. S6); and 3) depletion of cytokines is negligible (fig. S7), such that cytokine concentrations can be taken as constant on our experimental timescale. In this framework, a model for cytokine EC_{50} is determined by the set of possible receptor complexes formed, or equivalently by the set of affinity constants associated with their formation (see Supplementary Material). For this system, all of the receptors chains' abundance at the surface can be measured (fig. S8, Materials and Methods) and all the potentially required affinity constants have been experimentally determined, directly or indirectly (fig. S9) (20, 29, 31–36). Thus one should be able to create a model reproducing the data directly from these experimentally determined affinity constants. Based on the potential interactions between subunits of the IL-2 and IL-7 receptors, there exist 553 relevant models that can be enumerated systematically (fig. S10–S11, Supplementary Methods). However, none of the possible models quantitatively reproduce the positive correlation between IL-7 EC_{50} and IL-2R α abundance when affinity constants are fixed to their experimentally determined values (Fig. 3D, “Model with measured affinity constants”). The best among these poorly performing models is shown in fig. S12. Alternatively, when the affinity constants are unconstrained and free to vary, very good agreement between data and model output (Fig. 3D, “Model with free affinity constants”) can be obtained for a large number of models, but at the expense of fitted affinity constants that greatly deviate from their measured values (fig. S13).

This discrepancy can be resolved by acknowledging some intrinsic uncertainties in the experimental determination of affinity constants (see fig. S9) and translation of in vitro measurements to living cells. To do so, we introduced a Bayesian strategy to model the CCVA-measured EC_{50} values while relying on previous measurements of affinity constants as prior estimates of model parameters (see Supplementary Methods). This procedure allows the identification of a set of models that achieve a good agreement with the CCVA-measured EC_{50} values (Fig. 3D, “Model with constrained affinity constants”), while maintaining the affinity constants in a realistic range around their measured values (fig. S14–18). We relied on Akaike's information criterion (see Supplementary Material) to select the minimal model that accurately reproduces the CCVA data. This results in a model in which external cytokines bind only to the fully assembled receptors IL-7R α : γ_c and IL-2R α : β : γ_c , and the opposite correlation of IL-2 and IL-7 EC_{50} s with IL-2R α abundance can be readily interpreted as a consequence of the preformation of IL-2 receptors depriving IL-7 receptors of the γ_c (Fig. 3E). This prediction of the model could be tested and confirmed experimentally: we sorted effector T cells into highest and lowest 35% of IL-2R α abundance, and analyzed their steady-state association of γ_c , and IL-7R α by immunoprecipitation and western blot analysis (Fig. 3F). This blot demonstrated that IL-7R α : γ_c complexes were present in cells with low abundance of IL-2R α , but almost totally absent in cells with high abundance of IL-2R α .

An extended equilibrium binding model accounts for IL-2R α effects on other γ_c family cytokine EC₅₀s

IL-7 is only one among the γ_c family of cytokine whose receptors share some components with the IL-2 receptor. Among them, IL-4, -7, -15, and -21 all triggered pSTAT5 response in effector T cells, whereas IL-9 did not (fig. S19). Using CCVA, we found that the EC₅₀ to IL-4, -7, -15, and -21 do not behave identically as a function of IL-2R α abundance (Fig. 4A). Aside from the case of IL-2, the EC₅₀ of IL-7 response is the most strongly affected by the abundance of IL-2R α . For IL-15, a slight dependency can be observed whereas the EC₅₀ of IL-4 and IL-21 responses are largely unaffected by IL-2R α abundance. To account for the variety of these behaviors, we extended the simple model selected above to include these additional receptors (Fig. 4B), using typical protein abundances defined by flow cytometric calibration (fig. S8).

We modeled the full system using our previously estimated affinity constants related to the formation of the IL-2 and IL-7 receptors and adjusted the other affinity constants (fig. S20) to reproduce the effects of IL-2R α abundance on the EC₅₀ of the responses to these cytokines (Fig. 4C). We find that high affinity constants for the binding of γ_c to IL-21R and IL-4R α are required to recapitulate the independence of IL-4 and IL-21 EC₅₀ from IL-2R α abundance (fig. S20). Consequently, preformation of IL-4 and IL-21 receptors is largely unaffected by IL-2R α (Fig. 4D). To our knowledge, affinity constants for the binding of γ_c to IL-21R and IL-4R α have not been measured and our model predicts high values for these affinity constants on cell surfaces. Average EC₅₀ is tuned through the binding affinity of the preassembled receptor to the cytokine and abundance of IL-21R or IL-4R α . Similarly, in the case of IL-15, a high affinity binding between IL-15R α and $\beta:\gamma_c$ (fig. S20) leads to weak correlation of IL-15 EC₅₀ with IL-2R α abundance. In our model of the γ_c cytokine family, independence of a given cytokine EC₅₀ from IL-2R α abundance is achieved when the corresponding α chain has a high affinity for the shared component(s).

DISCUSSION

In this study, we have presented a comprehensive methodology to leverage the variation of protein abundance within cell populations in order to quantify variability in cytokine sensitivity and investigate its underlying mechanisms. This method relies on the demonstrated ability of flow cytometry to precisely measure the abundance of multiple proteins simultaneously in single cells (Fig. 1). We have used this methodology to study receptor signaling in the γ_c chain family, uncovering unexpected relationships between related receptors.

During activation of isogenic T cells, the abundance of IL-2R α varies by up to five orders of magnitude (17). Our co-staining experiments verified that these measurements of IL-2R α precisely define IL-2R α abundance in these cells (Fig. 1F, fig. S1). This allowed us to study the effect of this broad range of IL-2R α abundance on cytokine sensitivity, as defined by the EC₅₀ of STAT5 phosphorylation stimulated by the cytokine. In agreement with previous findings, we found that IL-2R α abundance strongly correlates with IL-2 sensitivity, with an EC₅₀ decreasing from 100pM to 1pM as IL-2R α abundance increases from 10² to 10⁷ proteins (Fig. 1, fig. S2). This is consistent with Cantrell & Smith's observation that cells endowed with higher IL-2R α abundance proliferate more robustly in vitro (22). Strikingly, we observed that this increase of IL-2 sensitivity is concomitant with a 20-fold decrease of IL-7 sensitivity (Fig. 3). The dependency of IL-7 sensitivity on IL-2R α abundance could not be explained by anti-correlation of IL-7R α and IL-2R α abundance or by a potential inhibition of IL-7R α signaling capacity as a function of IL-2R α abundance within activated effector cells (fig. S3–S4). Such anti-correlation of receptor abundance has, however, been observed both during T cells' transitions from naïve to effector and effector to memory

stages in vivo: naïve cells have abundant IL-7R α , but no IL-2R α ; memory cell precursors are marked by relatively high abundance of IL-7R α (25) and low abundance of IL-2R α (18). Additionally, we found that highly activated effector cells, with large amounts of IL-2R α , are inhibited from sensing IL-7: this implies that they would rely chiefly on IL-2 for survival. This could reinforce the suppressive effect of IL-2 depletion by T regulatory cells, preventing IL-7 from rescuing effector cells when IL-2 becomes scarce (17, 37). In this context, the inhibitory effect of IL-2R α abundance on IL-7 sensitivity represents an additional mechanism to enhance separation of cytokine specificities across different T cell subtypes, with naïve and memory cells relying chiefly on IL-7, while effectors rely on IL-2.

Addressing the impact of IL-2R α abundance on IL-7 sensitivity at the computational level was made possible because this system is well characterized at the biophysical level (20, 27, 29, 31–36). Binding interactions between receptor subunits, and between receptor subunits and cytokines have been quantified (29). However, because many of these measurements have been made in different systems and with varying methodologies—on the surface of cells or with soluble fractions—multiple models have been proposed for the function of these receptors (28). Our CCVA methodology (Fig. 2) allows us to add detailed quantitative data, across a large range of protein abundance, thereby placing tight constraints on any proposed model of IL-2 and IL-7 signaling. In the present work, we show that equilibrium binding models incorporating the measured affinity constants as priors quantitatively reproduce aspects of the dependency of IL-7 and IL-2 sensitivities on IL-2R α (Fig. 3). The switch of sensitivities between IL-2 and IL-7 as a function of IL-2R α abundance can be readily attributed to the preformation of the full heterotrimeric IL-2 receptor, which sequesters the γ_c chain away from the IL-7 receptor. By comparing all possible equilibrium binding models of formation of signaling cytokine-receptor complexes, we have identified a minimal equilibrium binding model with affinity constants constrained around the measured values that accounts quantitatively for the CCVA data. This model was selected using a statistical criterion comprising a cost for deviation from both biochemically measured parameters and CCVA data, and penalizing for inclusion of a larger set of parameters. As such, this selection procedure does not exclude other models with more parameters, representing other paths of receptor formation (35, 38, 39), but more data would be needed to justify their inclusion. The selected model presents the practical advantage of providing a straightforward interpretation of the scaling of IL-7 sensitivity as a function of IL-2R α abundance. The increased EC₅₀ at high IL-2R α abundance is a direct consequence of the sequestration of the γ_c chain in the preformed IL-2 receptor. As a consequence, a cytokine receptor subunit with a high affinity for a shared component (i.e. γ_c or β) should make it difficult for the IL-2 receptor to sequester this component, and the effect of IL-2R α abundance on the sensitivity to the corresponding cytokine should be weak.

Extending this minimal model to other cytokines of the γ_c family, we recapitulated different measured effects of IL-2R α on cytokine sensitivities (Fig. 4). Specifically, we found that IL-2R α abundance negatively impacts sensitivity to IL-7 and to a lesser extent to IL-15, but not at all to IL-4 or IL-21. In our model, non-dependence of sensitivity to IL-2R α for these two cytokines implies high affinity between the corresponding α chains and the shared component(s). The ability of each α chain to tune not only the sensitivity to its respective cytokine, but also cross-antagonism of other signals provides a functional separation between γ_c -utilizing cytokines that matches their respective functions in the context of T cell differentiation (40). IL-7 is dedicated to the homeostasis of naïve and memory cells, but not to the expansion of effectors, whereas IL-15 affects both effectors and memory cells (41). Therefore, segregating the homeostatic functions of IL-7 from the expansion function of IL-2 may be advantageous (24). In contrast, IL-4 and IL-21 are both involved in the differentiation of effector cells into helper subtypes, and are experienced during expansion

(21). For these two cytokines, cross-antagonism from large amounts of IL-2R α would thus be functionally counter-productive.

Our CCVA method can be applied to virtually any cellular signaling investigation wherein cellular heterogeneity and signaling can be measured, such as other cytokines, chemokines, or growth factors (42, 43). The number of signaling regulators that can be simultaneously measured is limited only by technical constraints of flow cytometry. As the limitations of flow cytometry recede with the introduction of machines capable of recording dozens of parameters simultaneously (10, 11), our method is poised to identify and quantify regulatory effects in signaling networks. This technical framework, accompanied by quantitative modeling efforts, will improve mechanistic understanding of signal transduction cascades in living cells (2, 3).

MATERIALS AND METHODS

Reagents and antibodies

Recombinant mouse IL-2, IL-4, IL-7, IL-12, IL-21, interferon gamma, and thymic stromal lymphopoietin (TSLP) were from eBioscience (San Diego, CA); IL-15 was from R&D systems (Minneapolis, MN). See table below for details of antibodies used.

Epitope	Clone	Conjugate	Usage	Supplier
CD4	RM4-5	Alexa-700	FC	eBio
IL-2R α (CD25)	PC61.5	PE-Cy7	FC	eBio
	PC61.5	Per-CP-Cy5.5	FC	eBio
	PC61.5	PE	FACS	Miltenyi
	7D4	FITC	FC	eBio
IL-7R α (CD127)	eBioSB/199	PE	FC	eBio
	P14	none	WB	scbt
γ_c (CD132)	4G3	PE	FC	eBio
	H-300	none	IP	scbt
IL-2R β (CD122)	TM- β 1	PE	FC	eBio
IL-4R α (CD124)	mIL4R-M1	PE	FC	BD
IL-21R	4A9	PE	FC	BD
IL-15-R α (CD125)	DNT15Ra	APC	FC	eBio
pY694-STAT5	C11C5	none	FC	Cell Signal
rabbit IgG	NA	APC	FC	Jackson IR
goat IgG	NA	HRP	WB	scbt

FC - Flow Cytometry, FACS - Fluorescence-Activated Cell Sorting, WB - Western Blot, IP - Immunoprecipitation

PE – Phycoerythrin, APC – Allophycocyanin, FITC – Fluorescein Isothiocyanate, HRP – Horseradish Peroxidase, Per-CP – peridinin chlorophyll protein, Cy - Cyanine

eBio - eBioscience, San Diego, CA

Miltenyi - Miltenyi Biosciences, Cambridge, MA

Cell Signal - Cell Signaling Technology, Danvers, MA

BD - BD Biosciences, San Jose, CA

scbt - Santa Cruz Biotechnology, Santa Cruz, CA

Jackson IR - Jackson Immunoresearch, West Grove, PA

Media

All in vitro experiments were performed in complemented RPMI medium (prepared by the Media facility at MSKCC), which consists of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 µg/ml of penicillin and 100 µg/ml of streptomycin and 50 µM β-mercaptoethanol. All cell cultures were maintained in an incubator at 37 °C with 5% CO₂.

Cell culture

5C.C7 TCR transgenic, Rag2^{-/-} IL-2^{-/-} CD4 T cells were isolated and expanded as described previously (13). Briefly, cells were expanded with irradiated B10A CD3^{-/-} splenocytes pulsed with 1 µM MCC K5 peptide: ANERADLIA YFKAATKF (GenScript, Piscataway, NJ). IL-2 (10 nM) was given at one and four days post stimulation, then every three days for maintenance of cultures. Cells were used between day 5 and 20 of culture, unless otherwise noted. Live cells were purified prior to analysis by separation on a Ficoll gradient (GE, Piscataway, NJ).

Cytokine titrations

Before measuring cytokine responses, bound IL-2 was stripped from cell surface by a 2 minute incubation in 0.1 M glycine buffer equilibrated at pH 4.0. After two washes in RPMI, cells were rested for 30 minutes at 37 °C (44). Cytokine titrations were prepared in 96-well V-bottom plates on ice. Cells (10⁵ per well) were added to cytokines and incubated for ten minutes at 37 °C before processing for flow cytometry.

Flow Cytometry

Cells were prepared for single cell staining following protocols optimized by the Nolan group (45). Briefly, cells were fixed for 10 minutes on ice in 1.6% paraformaldehyde followed by permeabilization on ice in 90% methanol. Cells were then washed twice in fluorescence-activated cell sorting (FACS) buffer (PBS with 4% FCS and 0.1% sodium azide) and stained intracellularly for phospho-STAT5, followed by incubation with labeled secondary antibody and conjugated antibodies to receptors (CD4 and IL-2Rα). All staining was performed at room temperature in FACS buffer. Before flow cytometric acquisition, cells were stained with 5 µM 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) for exclusion of dividing (G₂) and apoptotic (sub-G₁) cells. Flow cytometry acquisition was performed on a LSR-II cytometer (BD Biosciences, San Jose, CA).

Receptor abundance calibration

A standard curve correlating fluorescence as measured by flow cytometry to PE molecules was calculated using Bangs Laboratories (Fishers, IN) Quantum R-PE MESF beads. "Corrected median fluorescence intensity" (cMedFI) values were calculated by subtracting

the median fluorescence of unstained beads from the median fluorescence value of each PE standard. A linear regression of log cMedFI to log PE bead MESF values was used to establish a standard curve. To calibrate typical abundances of receptor chains, cells were stained with saturating concentrations (as established by titration) of the respective PE-labeled antibodies or a nonspecific PE-labeled rat IgG control. Corrected median fluorescence was calculated by subtracting the median fluorescence of the isotype control from the median fluorescence of each antibody staining. The calibration curve was used directly to calculate numbers of molecules from the corrected median fluorescence (fig. S8), using a ratio of 1 PE molecule per antibody, as specified by the manufacturer (eBioscience or BD Biosciences). For calibration of IL-2R α abundance in *ScatterSlice* data, the calibration curve was applied to each bin's fluorescence value.

Population sensitivity fitting

Population EC₅₀ values were determined from flow cytometry data, by fitting the dose response for the geometric mean of pSTAT5 as a function of the cytokine concentration with the following equation:

$$pSTAT5([cytokine]) = pSTAT5_{low} + \frac{pSTAT5_{high} - pSTAT5_{low}}{1 + \frac{EC_{50}}{[cytokine]}}$$

where $pSTAT5_{low}$ and $pSTAT5_{high}$ are the baseline and the plateau of pSTAT5 signals. Fits were performed with Prism (GraphPad Software; La Jolla, CA). The fitting algorithm uses the Hessian matrix (46) to estimate errors on the fitted parameters.

Fluorescence-Activated Cell Sorting (FACS)

Cells were Ficoll purified, then stained with PE- anti-CD25 (Clone PC61.5, Miltenyi Biosciences, Cambridge, MA) and APC-anti-CD4 on ice for 30 minutes. Prior to sorting, 5 μ M DAPI was added for exclusion of dead cells. Sorting was performed on a FACS Aria from BD Biosciences (San Jose, CA). Sorted cells were kept on ice until further processing.

Western Blotting

Equal numbers of sorted cells were rinsed in PBS, then lysed in buffer containing Tris HCl 25 mM, EDTA 1 mM, NaCl 130 mM, NP-40 1%; supplemented with protease inhibitor cocktail from Sigma Aldrich (St. Louis, MO) containing to final concentrations of 1.04 mM AEBSF, 800 μ M Aprotinin, 40 μ M Bestatin, 14 μ M E-64, 20 μ M Leupeptin, and 15 μ M Pepstatin A. Lysates were mixed with NuPAGE LDS loading buffer with 2.5% beta-mercaptoethanol, incubated at 95 °C for 2 minutes before loading and running on NuPAGE Novex 4–12% bis-tris gels (all products from Life Technologies, Grand Island, NY). Blots were transferred to Immobilon membrane (Millipore, Billerica, MA), prior to blotting with primary (IL-7R α) followed by secondary (anti-rabbit-IgG-HRP) antibodies in TBS with 0.05% Tween-20 and 2% BSA.

Immunoprecipitation of γ_c from sorted cells

T cells from (5×10^8) were stripped in 5 ml of 0.1 M glycine buffer equilibrated at pH 4.0. After two washes in RPMI, cells were rested for 30 minutes at 37 °C. Cells were then pelleted and resuspended in 10 ml fresh RPMI for staining, and processed for FACS as described above. 35% highest and lowest abundance IL-2R α fractions were collected. Following sorting, immunoprecipitation was performed using the Dynabeads co-immunoprecipitation kit (Life Technologies), following the manufacturer's protocol, with the following modifications. Cells were pelleted, weighed, and suspended in a 13:1 ratio of

Dynabeads lysis buffer (“Buffering Salts” pH 7.4, 110 mM potassium acetate, 0.5 % Triton X-100, 100 μ M sodium chloride). After incubation on ice for 20 min, cell lysates were passed through a 27-gauge syringe 10 times on ice. Immunoprecipitation was carried out according to the manufacturer's instructions, using Dynabeads freshly conjugated to anti- γ_c antibody (clone H-300). Western blotting was carried out as described above.

ScatterSlice analysis

Initial analysis of flow cytometry data was performed using FlowJo (Treestar, Ashland, OR). Single, CD4 positive cells with 2n DNA content were identified and exported as text files for analysis by our processing R program *ScatterSlice*.

ScatterSlice was written in R (47). The software is freely available upon request. Details of the algorithm are as follows:

Text files are imported into R, and then divided into user-specified bins. Within each bin, a three parameter Hill equation—fitting base, amplitude and EC_{50} —is fit by minimizing the sum across all doses in a dose-response (from $i = 1$ to N_{doses}) of the squared difference between the log of each dose's geometric mean fluorescence intensity of the response channel (pSTAT5 in our studies) (z_i^{obs}) minus the log of the three parameter hill equation (fitting *base*, *amplitude*, and EC_{50}), normalized by multiplying by the number of cells in that dose (N_i) divided by the phospho-response variance (σ_i^2). Errors on fitted parameters are estimated using the Hessian matrix (46). Tables of fitting results (values for base, amplitude, EC_{50} and their errors for different amounts of receptors) are presented as color maps and can be further analyzed with R or exported as text files for analysis with other software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. IL-2 EC₅₀s are anti-correlated with IL-2R α abundance

In vitro cultures of primary T cells were examined at day 3 (pink), 5 (green), 6 (orange) or 30 (blue) following initial in vitro activation and propagation in IL-2-supplemented medium. Cultures were selected from N=10 experiments to show range of responses. **(A)** Ten minutes following stimulation with 10 pM of IL-2 (colored histograms), T cells display variability in pSTAT5 distribution. pSTAT5 distributions were normalized between 2% and 98% of each culture's unstimulated and maximal responses, respectively. Gray histogram depicts control (unstimulated) pSTAT5 staining. **(B)** Hill equations (curves) fit to normalized dose responses (filled circles) display large variability in EC₅₀s (crosses), the characteristic concentration of IL-2 for which 50% of the maximal pSTAT5 response is reached. **(C)** EC₅₀ for IL-2 response varies over 2 orders of magnitude and is anti-correlated with abundance of IL-2R α . Black squares represent additional cultures of T cells not depicted in other panels. Error bars show error in EC₅₀ fitting. **(D)** Histograms of calibrated IL-2R α abundance for each of the culture under consideration. **(E)** Scheme for dual-labeling of IL-2R α . **(F)** Flow cytometry of cells singly labeled with PE-Cy7- (red) or FITC- (blue) conjugated antibodies to IL-2R α demonstrates a CV of 1.5. In cells stained with both antibodies simultaneously (purple), however, the ratio of the two stains has a CV of only 0.2.

Fig. 2. Cell-to-cell variability analysis (CCVA) methodology

(A) Overall methodology: Cells are first treated with a dose-response of a stimulus, which can be a cytokine, drug, or any treatment resulting in changes in cells measurable by flow cytometry. Following a short stimulation, cells are processed for flow cytometry. After acquisition by flow cytometry, data is processed through *ScatterSlice*, a user-friendly, graphical user interface for CCVA. **B–E: ScatterSlice internal processing.** **(B)** Binning the population for different amounts of IL-2Rα. Colors of dots are carried over to panels D and E. **(C)** The median value of the phospho-response, pSTAT5, is calculated within each IL-2Rα bin, for each dose. (Open histograms: no-cytokine control; red histograms: 10 pM IL-2). **(D)** Within each bin, across all doses of stimulus (IL-2), a curve with parameters of EC₅₀ and amplitude (solid lines) is fit across all IL-2 concentrations for which a sufficient number of cells exists within a bin to accurately estimate pSTAT5 (circles and dotted lines). **(E)** The output of the curve fitting is the relationship between IL-2Rα abundance and EC₅₀ (colored circles). The original histogram (gray) is shown for reference. Error bars show uncertainty in EC₅₀ fitting.

Fig. 3. Positive correlation between IL-7 EC₅₀ and IL-2Rα abundance: CCVA and modeling
(A) IL-7 EC₅₀ (red) and IL-2 EC₅₀ (blue) as a function of IL-2Rα abundance, measured by CCVA. Points show geometric mean ± SD of EC₅₀s from CCVA of two independent dose-responses with two replicates (this is representative of N=10 independent experiments). **(B)** Assembled cytokine/cytokine receptor complex for IL-2 and IL-7. **(C)** Schematic of the selected model: Closed circles – single components; open circles – multi-molecular complexes; colored circles – complete IL-2 (blue) and IL-7 (red) signaling complexes. A given complex sits on the line joining the two reactants involved in the corresponding binding reaction. **(D)** Comparison between the data and the fit for the model represented in (C) for different constraints on the parameters. Left: model output with measured parameters K_{exp}; center: fit with constraints; right: unconstrained fit **(E)** Modeled number of preformed IL-2 and IL-7 receptors (in the absence of external cytokine) as a function of IL-2Rα abundance, for the model represented in (C), with the parameters obtained from the constrained fit (D center). **(F)** Sorting of cells for highest and lowest 35% abundance of IL-2Rα, followed by immunoprecipitation (IP) using γ_c antibody and blotting for IL-7Rα shows diminished IL-7Rα in complex with γ_c in high IL-2Rα population.

Fig. 4. Extension of the preassembly model to full γ_c family

Experimental data (A) and model (B–D) of IL-2 (blue), IL-7 (red), IL-4 (brown), IL-15 (green) and IL-21 (purple) receptor formation. **(A)** Correlation of γ_c -family cytokine EC_{50} s with IL-2R α abundance, as measured by CCVA. Error bars represent geometric mean $\pm$ SD of EC_{50} s from CCVA of two independent replicate dose-responses; data shown is representative of 4 independent experiments. **(B)** Schematic of model incorporating all cytokine-specific α chains (see fig. S20 for the different parameters used). The single components are represented as closed circles; complexes are represented as open circles. A given complex sits on the line joining the two reactants involved in the corresponding binding reaction. The signaling cytokine-receptor complexes are colored depending on the cytokine involved. **(C)** Theoretical dependency of the sensitivity to various cytokines on IL-2R α abundance for the model shown. **(D)** Modeled number of preformed cytokine receptors (in the absence of external cytokine) as a function of IL-2R α . Note that the curve for IL-4 is shifted up slightly in order to distinguish it from the curve for IL-21.