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Abhinav Arneja, Hannah Johnson, Laura Gabrovsek, Douglas A. Lauffenburger and Forest M. White

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Qualitatively Different T Cell Phenotypic Responses to IL-2 versus IL-15 Are Unified by Identical Dependences on Receptor Signal Strength and Duration

Abhinav Arneja,*† Hannah Johnson,*† Laura Gabrovsek,‡ Douglas A. Lauffenburger,*†‡ and Forest M. White*,†

IL-2 and IL-15 are common γ-chain family cytokines involved in regulation of T cell differentiation and homeostasis. Despite signaling through the same receptors, IL-2 and IL-15 have non-redundant roles in T cell biology, both physiologically and at the cellular level. The mechanisms by which IL-2 and IL-15 trigger distinct phenotypes in T cells remain elusive. To elucidate these mechanisms, we performed a quantitative comparison of the phosphotyrosine signaling network and resulting phenotypes triggered by IL-2 and IL-15. This study revealed that the signaling networks activated by IL-2 or IL-15 are highly similar and that T cell proliferation and metabolism are controlled in a quantitatively distinct manner through IL-2/IL-15 signal strength independent of the cytokine identity. Distinct phenotypes associated with IL-2 or IL-15 stimulation therefore arise through differential regulation of IL-2/15R signal strength and duration because of differences in cytokine–receptor binding affinity, receptor expression levels, physiological cytokine levels, and cytokine–receptor intracellular trafficking kinetics. These results provide important insights into the function of other shared cytokine and growth factor receptors, quantitative regulation of cell proliferation and metabolism through signal transduction, and improved design of cytokine based clinical immunomodulatory therapies for cancer and infectious diseases. *The Journal of Immunology, 2014, 192: 000–000.

Interleukin-2 and IL-15 are critically involved in the regulation of peripheral T lymphocyte homeostasis and differentiation. IL-2 and IL-15 were among the first cytokines shown to trigger proliferation of activated T cells in vitro and in vivo (1, 2). Their ability to expand T cell numbers upon exogenous stimulation has made both cytokines extremely important in clinical settings as immunomodulatory and cancer immunotherapeutic agents (3–5). Both cytokines mediate their effects on T cells through a heterotrimeric receptor complex consisting of a cytokine specific α-chain (IL-2Rα or IL-15Rα), the common γ-chain (γc), and the IL-2/15R β-chain (β) (6–9). The α-chains function primarily as high-affinity ligand capture receptors while signal transduction occurs exclusively through the β and γc chains, which are constitutively associated with the JAKs (10). As a consequence of sharing the β and the γc chains, stimulation of T cells with IL-2 and IL-15 results in the activation of similar signaling pathways, which include the JAK-STAT, the Ras-Raf-MAPK, and the PI3K-Akt pathways (4, 10, 11). In addition, stimulation of T cells with IL-2 or IL-15 has been shown to result in the induction of similar gene expression profiles (8, 12, 13). Despite signaling through the same receptors and sharing common effector pathways, multiple studies have reported unique and even antagonistic roles for IL-2 and IL-15 in the T cell immune response. IL-2 is critical for the clonal expansion of activated T cells, differentiation of effector and memory cytotoxic T lymphocytes, and regulation of T cell peripheral tolerance (4, 14, 15). In contrast IL-15 is critically involved in the maintenance and survival of memory CD8+ T cells, naive CD8+ T cells, and NK cells. IL-2 and IL-15 stimulation of T cells can result in distinct phenotypic responses even at the cellular level (4, 16, 17). Ag-activated mouse CD8+ T cells cultured with IL-2 are metabolically more active and larger in size than cells cultured with IL-15 despite proliferating equivalently (18). In addition, a transient pulse of IL-15, but not IL-2, triggers T cell proliferation in an in vitro assay (19, 20).

Multiple factors may contribute to functional differences triggered by IL-2 and IL-15 stimulation of T cells. IL-2 and IL-15 differ in their mode of presentation to T cells. IL-2 directly binds IL-2Rα-chains expressed on T cells, whereas IL-15/IL-15Rα complexes on non-T cells are presented in trans to IL-2/15βγc complexes expressed on T cells in addition to directly binding IL-15Rα-chains expressed on T cells (4, 19, 21). Binding affinity of cytokines for their respective α-chains also may play an important role in differentiating the response to IL-2 and IL-15, because the binding affinity of IL-15 for IL-15Rα-chain is ~1000-fold higher compared with the affinity of IL-2 for IL-2Rα (19, 20). In support of this, IL-2 mutants engineered with significantly higher binding affinity for

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IL-2Rα trigger equivalent proliferation compared with IL-15 upon pulse stimulation of T cells (20). Signaling kinetics have also been implicated in differential regulation of T cell phenotype, because differences in cell size and metabolic activity between Ag-activated mouse CD8+ T cells cultured with IL-2 and IL-15 were associated with different kinetics of PI3K/PDK1 signaling triggered by the two cytokines (18). Although these studies have unveiled myriad possibilities for the distinct phenotypes resulting from stimulation with these two cytokines, the molecular mechanisms leading to differential regulation of T cell proliferation and metabolism through IL-2 and IL-15 remain incompletely characterized.

To elucidate the molecular mechanisms underlying the distinct T cell phenotypes driven by IL-2 and IL-15, we compared phosphotyrosine signaling networks triggered by the two cytokines and determined that the signaling networks activated by IL-2 and IL-15 are virtually identical. Because the disparate phenotypic response was not encoded in the signaling network, we focused on the role of IL-2/15R signal strength and duration in regulating cell proliferation and metabolic activity in engineered and primary human T cells. Our results indicate that the strength of signal is directly proportional to cellular metabolic activity and increase in cell size, whereas cell proliferation requires a constant signal above a threshold. Intriguingly, phenotypic regulation is independent of cytokine identity when presentation and duration are held constant. These results provide key insights into the differential regulation of cell proliferation and metabolic activity through shared signaling receptors that ultimately inform improved cytokine-based immunotherapies for the treatment of cancer, autoimmune disorders, and infectious disease.

Materials and Methods

Abs and Methods

Recombinant human IL-2 and IL-15 were purchased from PeproTech (Rocky Hill, NJ). High-affinity mutant IL-2 (mtIL-2) was a gift from K.D. Wittrup (David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA). JAK inhibitor 1 (J1) was purchased from EMD Millipore (Billerica, MA). CFSE and CellTrace Violet were purchased from Life Technologies (Grand Island, NY). Alexa Fluor 647–conjugated anti–IL-2Rα and allophycocyanin-conjugated anti–IL-2Rβ and anti–IL-15Rα mAbs were purchased from R&D Systems (Minneapolis, MN). Alexa Fluor 647–conjugated anti–phosphorylated (p)STAT5 (pY694) and anti–p66 (pS235/p236) Abs were purchased from BD Biosciences (San Jose, CA). Human anti-CD3ε (clone UCHT1) and human anti-CD28 (clone 37407) mAbs were purchased from R&D Systems (Minneapolis, MN).

Cell culture

F15R-Kit cell culture. F15R-Kit cells were a gift from the K.D. Wittrup. F15R-Kit cells were maintained at 37°C and 5% CO2 in RPMI 1640 medium supplemented with 10% FBS (heat inactivated), 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin–streptomycin, and 900 μg/ml G418. Unless otherwise indicated, cells were cultured in 80 pM IL-2 at a density of 2 × 106 cells/ml and passaged every 48 h.

Primary human T cell isolation and culture. PBMCs were isolated using Ficoll–Paque gradient centrifugation of unpurified human buffy coats (Recombinant Human Blood Components, Boston, MA). CD4+ and CD8+ T cells were cultured in medium alone were used as unstimulated controls. The lysates were incubated on ice for 20 min and then stored in –80°C.

Phosphotyrosine enrichment. Protein G–agarose (80 μl; EMD Millipore, Billerica, MA) beads were incubated with three phosphotyrosine Abs: 12 μg PT66 (Sigma–Aldrich, St. Louis, MO), 12 μg pY100 (CST, Beverly, MA), and 12 μg 4G10 (EMD Millipore), and 200 μl IP buffer (100 mM Tris, 100 mM NaCl, and 1% Nonidet P-40 [NP-40] [pH 7.4]) and the beads adjusted to pH 7.4. ITRAQ labeling. Peptide labeling with iTRAQ 8plex (AB Sciex, Framingham, MA) was performed as described previously. Briefly, for each analysis, ~400 μg (prior to desalting and processing) peptide for each condition was labeled with iTRAQ 8plex reagent. Lysylated peptides were dissolved in 30 μl 500 mM triethylammonium bicarbonate (pH 8.5), and the ITRAQ reagent was added and the mixture incubated for 8 h at 4°C with rotation. iTRAQ 8plex labeled peptides resuspended in IP buffer were added to Ab-conjugated protein G–agarose beads and incubated overnight at 4°C with rotation. Peptide-conjugated protein G–agarose beads were rinsed with 400 μl IP buffer and 4 × 400 μl rinse buffer (100 mM Tris [pH 7.4]), and peptides were eluted into 70 μl 100 mM glycine (pH 2.0). Phosphotyrosine peptides were further enriched using an offline immobilized metal affinity chromatography column. Peptides retained on the immobilized metal affinity chromatography column were loaded onto a C18 precolumn and were subsequently separated by reverse-phase HPLC (Agilent) over a 150-min gradient prior to nano-electrospray into an Orbitrap Elite mass spectrometer (Thermo Scientific) for phosphotyrosine analyses. To correct for slight variations in the amount of sample in each of the iTRAQ channels, the mean iTRAQ ratios for all proteins identified in each biological replicate analysis was used to normalize the data. The mass spectrometer was operated in data-dependent mode with a full-scan mass spectrometry (MS) spectrum, followed by tandem mass spectrometry (MS/MS) (collision induced dissociation was set at 35% energy for sequence information and higher energy c-trap dissociation at 75% energy for ITRAQ quantification for Orbitrap Elite) for the identification of precursor ions. Ion trap was operated at 35% for MS and precursor ions were allowed for 100 ms and Fourier transform–MS ion injection time was set to 1000 ms with a resolution of 60,000 across m/z 400–2000. For IT and FT-MS/MS scans, fragmentation was carried out on ions above a threshold of 500 counts and a Fourier transform–MS resolution of 7,500.

Phosphotyrosine data analysis. Raw mass spectral data files (.RAW files) were converted into .mgf file format using DTA/Support software (http://msquant.sourceforge.net/). All resulting MS/MS peak lists were searched against a National Center for Biotechnology Information Uniprot 2009 database containing Homo sapiens protein sequences (37,743 entries) using Mascot version 2.1.03 (Matrix Science). Trypsin enzyme specificity was applied with a maximum of 1 missed cleavage. Mass tolerance for precursor ions was set to 10 ppm, and fragment ion mass tolerance was 0.8 Da. MS/MS spectra searches incorporated fixed modifications of carbamidomethylation of cysteine and iTRAQ 8plex modification of lysine and peptide N-termini. Variable modifications were oxidized methionine and phosphorylation of serine, threonine, and tyrosine residues. Phosphotyrosine peptides initially were filtered using an arbitrary mass spectrum score cutoff of 25. Precursor ions were manually evaluated, and peptides with contaminating peaks present within the isolation window (ions with intensity > 25% of the base peak ± 1.5 m/z) were discarded as the precursor ion m/z could not contribute to the relative iTRAQ intensities. iTRAQ intensity values were extracted from higher energy c-trap dissociation scans using an in-house python script that converted ITRAQ intensities into .txt format. We further imported this into Microsoft Excel, and iTRAQ values were isotope corrected based on the iTRAQ8plex correction matrix (AB
Sciex). Phosphotyrosine peptide iTRAQ ratios were normalized based on the mean relative protein quantification ratios obtained from the total protein.

### Western blotting

F15R-Kit cells were lysed on ice with radio IP assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with phosphatase and protease inhibitor (Thermo Scientific). The lysates were cleared through centrifugation at 10,000 × g for 10 min at 4˚C and then incubated with laemmli sample buffer at 95˚C for 5 min. Sample protein concentrations were determined using the bicinchoninic acid protein assay (Thermo Scientific). The samples were separated via PAGE using 10% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were probed overnight at 4˚C with primary Ab and then washed and stained with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary Abs. The blots were developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific). Radio IP assay buffer and laemmli sample buffers were purchased from Bio-Rad (Hercules, CA). Precautionary 10% Tris-HCl polyacrylamide gels were purchased from Bio-Rad (Hercules, CA). Anti-phosphoSTAT5 (pY694), anti-STAT5, anti-phosphoERK, anti-ERK, and anti-β-actin Abs were purchased from Cell Signaling Technology (Beverly, MA).

### Flow cytometry analysis

Cell surface staining was performed according to the manufacturer’s recommended protocol for the particular Ab used. PE-conjugated Abs against human IL-2Rα, IL-15Rα, IL-2Rβ, and IL-2Rγ proteins were purchased from R&D Systems (Minneapolis, MN). Alexa 647-conjugated Ab against human 40S ribosomal protein S6 phosphorylated at Ser235 and Ser236 were purchased from BD Biosciences (San Jose, CA). For intracellular staining, cells were washed and fixed in 1:1 dilution of PBS and BD Cytofix Buffer for 15 min at room temperature. Fixed cells were permeabilized on ice for 30 min in BD Phosflow Perm Buffer III and washed and stained with fluorochrome-conjugated Ab (5 × 10^5 cells in 50 μl) at 4˚C for 60 min. Samples were analyzed using either BD Accuri C6 cytometer or BD LSRFortessa cell analyzer (BD Biosciences), and data were analyzed using the FlowJo software (Tree Star, Ashland, OR). Flow cytometry events were acquired ungated, and live lymphocyte gates were created, based on their forward and side light scatter profiles using FlowJo.

### Proliferation assays

Cell counting using Trypan blue exclusion. F15R-Kit cells cytokine starved for 48 h were resuspended in fresh media at a density of 2 × 10^5 cells/ml and then cultured in the presence or absence of cytokines. For the pulse

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**FIGURE 1.** Mass spectrometry based phosphotyrosine profiling of F15R-Kit cells stimulated with IL-2 and IL-15. (A) Schematic diagram of MS-based quantitative phosphotyrosine profiling experimental workflow. (B) Heat maps depicting 85 phosphorylation sites on 81 proteins quantified across F15R-Kit cell stimulated with IL-2 or IL-15 for 15 min. Heat maps represent log_2-transformed iTRAQ-8plex fold change values relative to one replicate of unstimulated F15R-Kit cells. The fold changes depicted for IL-2 and IL-15 are an average of three technical replicates. (C) Cellular processes regulated by the 39 proteins with >1.54-fold increase in phosphorylation level upon IL-2 and IL-15 stimulation. (D) The relative increase in phosphorylation levels of canonical IL-2/15R signaling proteins triggered by IL-2 and IL-15 in F15R-Kit cells.
bioassay, cytokine-starved F15R-Kit cells were stimulated with cytokine for 30 min, washed three times, and resuspended in cytokine-free culture medium and maintained at 37°C and 5% CO₂. Viable cell numbers were determined through Trypan blue exclusion using the Vi-CELL Cell Viability Analyzer (Beckman Coulter).

**CFSE and CellTrace Violet staining.** Cells were labeled with CFSE or CellTrace Violet dyes, according to the manufacturer’s protocols, at a final concentration of 0.5–2.5 and 5 μM, respectively. CFSE and CellTrace Violet fluorescence levels were measured using the BD Accuri C6 cytometer and the BD LSRFortessa analyzer, respectively.

**Cell size and proliferation experiments**

**F15R-Kit cells.** CFSE-labeled cytokine-starved F15-Kit cells were seeded at a density of 2 × 10⁵ cells/ml and pretreated with the indicated doses of JI for 30 min at 37°C. The cells were then cultured with 500 PM IL-2 or IL-15 at 37°C and 5% CO₂ for 6 d.

**Primary human CD4⁺ and CD8⁺ T cells.** Purified CD4⁺ and CD8⁺ human primary T cells were labeled with CFSE (2.5 μM) and added to 12-well tissue culture plates coated with 10 μg/ml anti-CD3 mAb, supplemented with soluble anti-CD28 mAb (0.5 μg/ml), and cultured at 37°C and 5% CO₂ for 72 h. Cells cultured on uncoated plates in the absence of anti-CD28 served as unstimulated controls. Activated cells were washed, suspended in fresh medium, and cultured with the indicated dose of JI and 1 nM IL-2, 1 nM IL-15, or medium alone for 4 d.

Cells were passaged and cytokine and inhibitor levels were replenished every 48 h. Forward light scatter (FSC), CFSE dilution, and pSTAT5 levels were measured using the BD Accuri C6 cytometer at the indicated time points after cytokine addition.

**Glucose and lactate measurements**

F15R-Kit cells were cultured for 6 d with the indicated dose of JI and 500 pM IL-2 or IL-15. On day 6, cells were washed and seeded at a density of 2 × 10⁵ cells/ml in fresh medium, inhibitor, and cytokine. Culture media were collected 48 h later, and glucose and lactate levels were measured using the YSI 7100 Select Biochemistry Analyzer (YSI, Yellow Springs, OH).

**Statistical analysis**

Mean iTRAQ fold changes of individual phosphotyrosine sites generated through liquid chromatography (LC)-MS/MS analysis were compared using unpaired student’s t test. A p value < 0.05 was considered statistically significant. For multiple comparison testing, one-way ANOVA was performed followed by unpaired Student t tests for comparison of individual populations. To account for the presence of type I error because of multiple hypotheses testing, p values used for the determination of statistical significance were adjusted using the false discovery rate control method to correspond to a particular family-wise error rate (αfw). The p values less than the adjusted p values corresponding to αfw of 0.05 were considered significant.

**Results**

**Phosphotyrosine profiling of IL-2– or IL-15–stimulated T cells reveals the activation of highly similar signaling networks**

To quantitatively compare IL-2– and IL-15–mediated signal transduction in T cells at a network level, differentially iTRAQplex-labeled tyrosine-phosphorylated peptides from F15R-Kit cells stimulated for 15 min with no cytokine, IL-2, or IL-15 were immunoprecipitated with pan-specific anti-phosphotyrosine Abs and analyzed using reversed-phase LC-MS/MS (Fig. 1A) (22–25). The choice of F15R-Kit cells, a human leukemia–derived T cell line, as a model system was based on constitutive and stable expression of all four receptors of the IL-2/15R system in these cells (Supplemental Fig. 1A, 1B) (26). Phosphotyrosine profiling analysis led to the identification and quantification of 85 unique phosphor-
ylation sites (76 pTyr and 9 pSer/Thr) on 81 proteins measured across three biological replicates (Fig. 1B). To determine the minimum magnitude of difference detected through LC-MS/MS analysis in the current study, we analyzed the relative SD (through calculation of the average coefficient of variation) across all conditions and biological replicates. Using twice the relative SD as a cutoff, we defined a 1.54-fold change in iTRAQ values as the minimum difference reliably detected through MS analysis in the current study. It is important to note that the relative SD for the LC-MS/MS analysis is a combination of both the technical variations of LC-MS/MS and cell culture techniques and the inherent biological noise of our cell culture system. Consequently, the minimum difference threshold of 1.54-fold is specific to the present analysis and does not represent the sensitivity of the LC-MS/MS technique by itself. Of 85 total phosphorylation sites, 41 sites (38 pTyr and 3 pSer/Thr) from 39 proteins showed a 1.54-fold or higher increase in phosphorylation level upon IL-2 or IL-15 stimulation. The IL-2– and IL-15–responsive phosphorylation sites belonged to proteins involved in the regulation of multiple cellular processes including cytoskeletal regulation, endocytosis, and protein translation, in addition to proteins known to be involved in signal transduction (Fig. 1C, Supplemental Table 1). Only 2 of the 39 IL-2– and IL-15–responsive proteins showed quantitatively distinct tyrosine phosphorylation responses (i.e., >1.54-fold change) between IL-2 and IL-15 stimulation. IL-2 stimulation resulted in ~2.7-fold increase in phosphorylation of signal transducing adaptor molecule 2 (STAM2) on Tyr974 and a 2.0-fold increase in phosphorylation of vacuolar protein sorting 18 (VPS18) on Tyr74 relative to IL-15 stimulation (Fig. 2A). Both of these proteins have been shown to be involved in protein trafficking, endosomal sorting, and endocytosis (Supplemental Table 1) (27–30).

Overall IL-2 or IL-15 stimulation resulted in an equivalent increase in phosphorylation levels of canonical IL-2/15R signaling pathway proteins, confirming previous reports (Figs. 1D, 2B). Beyond canonical signaling pathways, our analysis demonstrated that stimulation with IL-2 or IL-15 resulted in equivalent tyrosine phosphorylation of many proteins not known to be directly involved in IL-2/15R signaling, indicating that the signaling response to IL-2 and IL-15 is similar throughout the network.

**F15R-Kit cells proliferate equivalently in response to continuous stimulation with IL-2 and IL-15 but differ in their response to pulse stimulation**

To understand how virtually identical signaling networks could give rise to qualitatively different phenotypic responses to IL-2 or IL-15 stimulation, we probed potentially revealing quantitative characteristics of receptor signaling and resultant cell phenotypic responses. To identify differential proliferation responses to the two cytokines, we counted viable F15R-Kit cell numbers following continuous stimulation with IL-2 or IL-15 over 5 d. F15R-Kit cells cultured in the continuous presence of IL-2 or IL-15 proliferated equivalently (Fig. 3A). To account for the effects of cytokine α-chain binding affinity on cell proliferation, F15R-Kit cells were cultured with an mtIL-2 engineered to have a binding affinity for IL-2Rα approaching that of the IL-15–IL-15Rα complex. Proliferation of F15R-Kit cells cultured with mtIL-2 was quantitatively equivalent to cells cultured with either IL-2 or IL-15 (Fig. 3A). To compare the proliferative response of F15R-Kit cells pulsed stimulated with IL-2, IL-15, or mtIL-2 for a defined 30 min, we used the pulse bioassay protocol described previously (20). Consistent with previous observations, F15R-Kit cells pulsed with IL-15 or mtIL-2 proliferated over a period of days without additional cytokine stimulation, whereas wild-type IL-2–pulsed cells did not proliferate (Fig. 3B).

**Increased initial receptor occupancy and surface persistence of cytokines after pulse stimulation mediates proliferation through increased duration of IL-2/15R signaling after cytokine withdrawal**

The proliferative response of F15R-Kit cells to cytokine pulse stimulation previously was found to be directly correlated to the initial cytokine surface receptor occupancy and surface persistence (20). We hypothesized that increased initial receptor occupancy and surface persistence may regulate proliferation through increased duration of IL-2/15R signal transduction after cytokine withdrawal. To test this hypothesis, the surface cytokine levels, IL-2/15R signal strength, and proliferation of F15R-Kit cells pulsed with IL-2, IL-15, or mtIL-2 were measured simultaneously at multiple time points after cytokine withdrawal. Because STAT5 proteins are tyrosine phosphorylated directly through association with the IL-2RβR, intracellular levels of pSTAT5 represent a proxy for IL-2/15R signal strength (10). F15R-Kit cells pulsed with IL-15 or mtIL-2 showed higher levels of surface-bound cytokine and surface persistence after cytokine withdrawal compared with wild-type IL-2 (Fig. 4A, 4B). Pulse stimulation of
F15R-Kit cells with IL-2, IL-15, or mtIL-2 initially resulted in a quantitatively equivalent increase in pSTAT5 levels (Fig. 4A). Cells pulsed with IL-15 or mtIL-2 maintained high pSTAT5 levels for up to 72 h after cytokine withdrawal, whereas the pSTAT5 levels of cells pulsed with wild-type IL-2 returned to basal levels by 6 h (Fig. 4C, Supplemental Fig. 2A, 2B).

**FIGURE 4.** F15R-Kit cell proliferation requires continuous activation of the IL-2/15R signaling. (A) Representative flow cytometry histograms showing surface cytokine and intracellular pSTAT5 levels in F15R-Kit cells stimulated with 500 pM of the indicated cytokine for 30 min (solid histograms). Dotted histograms represent unstimulated cells. Surface-bound cytokine levels (B) and intracellular pSTAT5 levels (C) of F15R-Kit cells, pulse stimulated with 500 pM cytokine measured at the indicated time points after cytokine withdrawal. Data represent median fluorescence intensity (MFI) of surface cytokine and pSTAT5 levels normalized to MFI of cells stimulated with IL-15 at the 30-min time point. (D) CFSE dilution values (left panel) and representative histograms of F15R-Kit CFSE levels (right panel) experiencing a defined duration of IL-2/15R signaling. ***p<0.001 compared with unstimulated cells. CFSE dilution values represent the ratio of CFSE MFI levels of cells cultured with IL-2 and JI to CFSE MFI of unstimulated cells 48 h after cytokine addition. Solid histograms represent CFSE values for F15R-Kit cells cultured with IL-2 and JI, and dashed histograms represent CFSE values of unstimulated cells 48 h after cytokine addition. Data represent average ± SD for three independent experiments.
**F15R-Kit cell proliferation requires a continuous signal input from the IL-2/15R**

F15R-kit cells pulsed with wild-type IL-2 maintained high pSTAT5 levels for up to 6–8 h after cytokine withdrawal yet did not proliferate. We hypothesized that a minimum duration of IL-2/15R signaling might be required for T cell proliferation. To test this hypothesis, CFSE-labeled F15R-Kit cells were cultured with a saturating IL-2 dose, and a saturating dose of JI was added at multiple time points after cytokine addition. Saturating doses were defined as the cytokine dose that induced maximal STAT5 phosphorylation and the JI dose that resulted in maximum reduction in pSTAT5 levels after cytokine stimulation. The addition of JI at any time point after cytokine stimulation allowed experimental control over the duration of IL-2/15R signaling experienced by the F15R-Kit cells. The addition of JI at any time point up to 30 h after IL-2 addition prevented F15R-Kit cells from undergoing cell division (Fig. 4D). These results suggest that continuous signal transduction from the IL-2/15R is required for T cell proliferation.

**F15R-Kit cell proliferation, cell size, and glycolytic activity respond distinctly to IL-2/15R signal strength**

To investigate the role of IL-2/15R signal strength in the regulation of T cell proliferation and cell size, CFSE-labeled F15R-Kit cells were cultured with increasing doses of IL-2 or IL-15; for each condition, cell proliferation and cell size were measured over multiple days. F15R-Kit cell size increased with increasing cytokine dose (Fig. 5A, 5C), but proliferation remained equivalent for IL-2– or IL-15–stimulated cells (Fig. 5B, 5C). Because the relationship between cytokine dose, cell size, and cell proliferation was identical for both IL-2 and IL-15, we hypothesized that T cell size and proliferation were regulated directly through the IL-2/15R signal strength in a cytokine-independent manner. To test this hypothesis, CFSE-labeled F15R-Kit cells were cultured with a saturating dose of IL-2 or IL-15 along with a range of JI doses, allowing for direct control of the IL-2/15R signal strength upon IL-2 or IL-15 stimulation (Fig. 6A). For either IL-2 or IL-15 stimulation, F15R-Kit cell size decreased with increasing JI concentration (Fig. 6B), whereas proliferation remained unaffected (Fig. 6C). The relative differences in F15R-Kit cell size and the equivalent relative F15R-Kit proliferation levels were maintained for up to 6 d in culture (Fig. 6B, 6C).

Increase in T cell size because of Ag or cytokine stimulation is related to increased cellular metabolic activity (31–34). To determine whether an increase in F15R-Kit cell size with IL-2/15R signal strength was also correlated with an increase in cell metabolism, we measured glucose consumption and lactate production by F15R-Kit cells maintained in culture with IL-2 or IL-15 in the presence of varying doses of JI. Glucose consumption and lactate production decreased with increasing JI dose for cells stimulated with either IL-2 or IL-15 (Fig. 6D, 6E), suggesting that metabolic activity is dependent on signal strength from the receptor, independent of cytokine identity.

**Regulation of F15R-Kit cell size and proliferation through the IL-2/15R signal strength is quantitatively distinct and cytokine independent**

To directly establish the quantitative relationship between signal strength, cell size, and proliferation, CFSE-labeled F15R-Kit cells were cultured with IL-2 or IL-15 along with a range of JI doses. FSC, CFSE dilution, and intracellular pSTAT5 levels were measured through flow cytometry after 48 h in culture. CFSE dilution levels initially decreased with increasing pSTAT5 levels but plateaued independent of further increase in pSTAT5 levels beyond 40% of maximal pSTAT5 levels triggered by cytokine stimulation, suggesting a digital relationship between cell proliferation and IL-2/15R signal strength (Fig. 7A). In contrast to CFSE dilution,
F15R-Kit FSC values increased linearly with respect to pSTAT5 levels, suggesting an analog relationship between cell size and signal strength (Fig. 7B). The PI3K/AKT/mTOR pathway has been shown to play a direct role in the regulation of T cell size and metabolic activity, and serine phosphorylation of 40S ribosomal protein S6 (pS6) is a reliable indicator of this pathway’s activation (18, 35, 36). Plotting F15R-Kit cell FSC values with respect to pS6 levels revealed an analog relationship between F15R-Kit cell size and mTOR pathway activation (Fig. 8), similar to our results for pSTAT5.
To determine whether IL-2/15R signal strength measurements alone were sufficient for predicting F15R-Kit cell size and proliferation in response to cytokine stimulation, CFSE-labeled F15R-Kit cells were cultured with IL-2 or IL-15 along with three different doses of JI. The stimulation, processing, and analysis of the varying cytokine and JI treatments were performed simultaneously to minimize variations introduced through the experimental techniques. Consistent with earlier results, the FSC of F15R-Kit cell size increased linearly with receptor signal strength (Fig. 7C, Supplemental Fig. 2C), but proliferation remained identical (Supplemental Fig. 2C). Data in Fig. 7C demonstrate that IL-2/15R signal strength was not only sufficient but a superior indicator of F15R-Kit cell size compared with cytokine identity, cytokine dose, or cytokine concentration.

The quantitative relationship between cell size, proliferation, and IL-2/15R signal strength is conserved in Ag-activated primary human T lymphocytes

To determine the physiological relevance of the quantitative relationship between IL-2/15R signal strength, cell size, and proliferation discovered using F15R-Kit cells, we replicated the experiments with primary human CD4+ and CD8+ T lymphocytes. Primary T cells were activated in vitro using anti-CD3 and anti-CD28 stimulation, which led to an upregulation of all four IL-2/15R chain surface levels (Supplemental Fig. 1C). Similar to F15R-Kit cells, IL-2– and IL-15–stimulated CD4+ and CD8+ T cells decreased in size with increasing JI dose (Fig. 9A, 9C), whereas proliferation levels remained unaffected (Fig. 9B, 9D). Differences in cell size were maintained for multiple days in cell culture, during which time the cell proliferation rates remained constant (Fig. 9A–D). Similar to F15R-Kit cells, plotting FSC and CFSE dilution levels of the primary human T cells with respect to intracellular pSTAT5 levels as a proxy for IL-2/15R signal strength revealed an analog relationship with cell size and a digital relationship with cell proliferation for both CD4+ and CD8+ T lymphocytes (Fig. 9E–H).

Discussion

IL-2 and IL-15 signal through the same receptors on the surface of T cells yet play distinct roles in the T cell immune response physiologically and at the cellular level. Results from the current study provide new insights into mechanisms by which IL-2 and IL-15 trigger distinct phenotypes in T cells.

To generate an unbiased, global, and quantitative view of the signal transduction triggered by IL-2 and IL-15 stimulation in T cells, we quantified changes in the pTyr proteome triggered in F15R-Kit cells upon IL-2 and IL-15 stimulation (22–25). With the exception of a quantitative difference in STAM2 and VPS18.
phosphorylation, IL-2 and IL-15 stimulation resulted in qualitatively and quantitatively identical signal transduction through the IL-2/15R in F15R-Kit cells. These results suggest that phenotypic differences induced by IL-2 and IL-15 in T cells are likely not a result of distinct signal transduction from the shared IL-2/15R. Interestingly, both STAM2 and VPS18 proteins are known to be involved in the regulation of cytokine and growth factor receptor endocytosis and intracellular trafficking (27–30). The effects of potential differences in receptor intracellular trafficking on cellular phenotypes remain to be characterized.

Results from our investigation into the distinct proliferative responses of T cells to pulsed stimulation with IL-2 or IL-15 revealed a critical role of IL-2/15R signal duration in regulating T cell proliferation. We found that the increased receptor occupancy and surface persistence of IL-15 and mIL-2, a result of their higher α-chain binding affinity, drove proliferation through prolonged duration of signaling from the receptor after cytokine withdrawal compared with cells pulsed with wild-type IL-2. A closer examination of the inability of F15R-Kit cells pulse stimulated with wild-type IL-2 to proliferate despite signaling for ~6 h revealed that T cell proliferation required a constant signal input from the IL-2/15R. This result may be related to the role of IL-2 signaling in T cell peripheral tolerance, where a requirement for constant IL-2/15R signaling for cell division might ensure proliferation of activated T cells only during the early immune response when high levels of IL-2 are produced and secreted by Ag-activated T lymphocytes (3, 4, 15, 37).

Investigation into the distinct metabolic activity triggered by IL-2 and IL-15 in T cells revealed the critical role played by IL-2/15R signal strength in the regulation of T cell proliferation and metabolism. We found that F15R-Kit cell size increased with IL-2/15R signal strength, whereas proliferation remained identical for both IL-2- and IL-15-stimulated cells. Because the relative differences in cell size were maintained over several days while proliferation rates remained constant, the cell size differences were not a result of differential rates of cell cycle progression. This result suggests that cell proliferation and size were both directly regulated through IL-2/15R signal strength but in a quantitatively distinct manner. F15R-Kit cell proliferation changed digitally with IL-2/15R signal strength, whereas F15R-Kit cell size showed an analog linear dependence on IL-2/15R signal strength.

The regulation of T cell size by IL-2/15R signal strength appears to be mediated through its effects on T cell metabolic activity, because glycolytic activity of F15R-Kit cells correlated directly with IL-2/15R signal strength and cell size. Proliferation remained identical for cells with glycolytic activity above a minimum threshold, suggesting that cytokine stimulation triggers metabolic activity in excess of proliferative demand. Although not directly measured in this study, the increase in glycolytic activity of F15R-Kit cells upon IL-2 or IL-15 stimulation is likely a result of increased expression and activity of glucose transporters and metabolic enzymes (31–35, 38). The analog relationship between glycolytic activity and consequently cell size may be a result of the direct dependence of glucose transporter and glycolytic enzyme expression levels on IL-2/15R signal strength (35, 36, 38, 39). Our results showing a linear relationship between cell size and mTOR pathway activation for both IL-2- and IL-15-stimulated F15R-Kit cells lend support to this idea (36, 38, 39).

Our experiments with primary human CD8+ and CD4+ T cells showed that the quantitatively distinct and cytokine independent regulation of T cell size and proliferation is a physiologically conserved mechanism for IL-2– and IL-15–mediated regulation of distinct T cell phenotypes. To our knowledge, this is the first time a stable analog relationship between IL-2/15R signal strength and cell size has been demonstrated for primary human CD8+ and CD4+ T lymphocytes. Interestingly, the significant difference in cell size upon IL-2 or IL-15 stimulation previously reported in mouse CD8+ T cells was not observed for either CD8+ or CD4+ human T lymphocytes (18). It is possible that activated human T cells express a higher amount of IL-15Rα on their surface compared with activated murine T cells either because of inherent species specific differences or distinct stimulation protocols used (anti-CD3/CD28 for humans versus antigentic peptides for mice). The higher IL-15Rα expression of human T cells would result in similar IL-2/15R signal strength and consequently cell size for cells stimulated with IL-2 and IL-15.

Recent studies in mouse models have revealed that IL-2 and IL-15 can differ in their mode of presentation in vivo. Even though both IL-2 and IL-15 stimulate T cells as soluble ligands (4, 9), IL-15 is thought to be mainly presented through a process known as trans-presentation (4, 8, 19, 21). This mode of presentation involves the interaction of IL-15–IL-15Rα complexes expressed on the surface of dendritic cells and macrophages with the IL-2/15Rbg complexes expressed on T cells (4, 8, 19, 21). Although trans-presentation was not present in our system, results from the current study can provide insights into its effects on IL-15 signaling. Because IL-15–IL-15Rα complexes presented in trans can directly stimulate the IL-2Rαβγc complexes independent of the T cell surface expression of IL-15Rα, and no known evidence exists of their ability to be internalized by the stimulated cells, trans presentation can potentially increase the strength and duration of IL-15 signal transduction relative to stimulation by soluble IL-15 (4, 18, 19, 40). Our current results suggest that T cells stimulated with IL-15 in trans would be metabolically more active, larger in size, and more proliferative compared with cells stimulated with soluble IL-15. In support of this idea, stimulation...
FIGURE 9. Quantitatively distinct regulation of cell size and proliferation through the IL-2/15R signal strength in primary human T lymphocytes. Primary human CD4+ and CD8+ T cells were activated with anti-CD3 and anti-CD28 stimulation, treated with the indicated JI dose and stimulated with 1 nM IL-2 or IL-15. FSC of CD4+ T cells (A) and CD8+ T cells (C) at the indicated time points after cytokine addition. CFSE dilution values for CD4+ T cells (B) and CD8+ T cells (D) at the indicated time points after cytokine addition. Nonactivated T cells were used as the negative control for measuring CFSE dilution. FSC versus intracellular pSTAT5 levels at 48 h after cytokine addition for CD4+ T cells (E) and CD8+ T cells (black squares) (F). CFSE dilution versus intracellular pSTAT5 levels at 48 h after cytokine addition for CD4+ T (gray circles) cells (G) and CD8+ T (black squares) cells (H).
of T cells with soluble complexes of IL-15–IL-15Rα results in stronger and more persistent signaling and a more potent phenotypic response compared with stimulation with soluble IL-15 (40, 41). It is possible that soluble and trans-presented IL-15 play distinct roles physiologically. Soluble IL-15 may primarily function to provide viability and proliferation signals for T cell populations that express IL-15Rα-chains on their surfaces, for instance, in the context of processes such as maintenance and self-renewal of memory T cells. Alternatively, trans-presented IL-15 may primarily function to provide a spatially localized “growth factor” like signal stimulating both metabolic activity and proliferation of T cells independent of IL-15Rα expression, which may be critical for the generation and maintenance of tissue localized effector T cell populations.

In summary, the current study investigated mechanisms allowing IL-2 and IL-15 to mediate distinct phenotypes in T cells despite signaling through the same receptors. We found that the strength and duration of a signaling pathway can directly affect cellular phenotypes, and regulation of distinct phenotypes may not require activation of qualitatively distinct signaling pathways. These results may explain the ability of cytokines to regulate different phenotypes in T cells while signaling through shared receptors. In support of this, a recent study explained differences in IL-7–elicited viability and proliferation behavior among various mouse strain naive CD8+ T cells in terms of analogous quantitative dependences of IL-7R signaling strength and duration (42). Because T cell effector functions and differentiation are intimately linked to their metabolic activity, the quantitative regulation of T cell metabolism and proliferation described in the current study may provide important design principles for improved cytokine mediated immunotherapies (3–5, 18, 32). Engineering mutant versions of the IL-2 receptor β and γ chains of the IL-2 receptor β chain by the novel cytokine IL-15. EMBO J. 13: 2822–2830.


