Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15

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Interleukin 15 (IL-15) and IL-2 have distinct immunological functions even though both signal through the receptor subunit IL-2Rβ and the common γ-chain (γc). Here we found that in the structure of the IL-15–IL-15Rα–IL-2Rβ–γc quaternary complex, IL-15 binds to IL-2Rβ and γc in a heterodimer nearly indistinguishable from that of the IL-2–IL-2Rα–IL-2Rβ–γc complex, despite their different receptor-binding chemistries. IL-15Rα substantially increased the affinity of IL-15 for IL-2Rβ, and this allostery was required for IL-15 trans signaling. Consistent with their identical IL-2Rβ–γc dimer geometries, IL-2 and IL-15 showed similar signaling properties in lymphocytes, with any differences resulting from disparate receptor affinities. Thus, IL-15 and IL-2 induced similar signals, and the cytokine specificity of IL-2Rα versus IL-15Rα determined cellular responsiveness.

Our results provide new insights for the development of specific immunotherapeutics based on IL-15 or IL-2.

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actions of IL-2 and IL-15, whether functionally or structurally derived, are not mutually exclusive, and the extent to which each contributes to the unique biological effects of IL-2 and IL-15 is unclear.

In this report we investigate several aspects of IL-15 structural and functional biology. First, we determined the crystal structure of the IL-15 quaternary complex to compare the molecular recognition strategies used by IL-15 and IL-2 in binding the shared IL-2Rβ and γc subunits, as well as to assess the relative geometries of receptor heterodimerization induced by the two cytokines. Second, informed by those structural comparisons, we did molecular dynamics simulations and biophysical affinity measurements to probe the mechanisms whereby IL-15Rα enhances the potency of IL-15. Finally, given the structural data indicating very similar receptor-binding modes, we characterized the signaling and gene-expression profiles of lymphocytes induced by IL-2 and IL-15 to assess whether these cytokines produce different intracellular signals that could explain their functional differences.

RESULTS

Comparison of the IL-15 and IL-2 quaternary complexes

Our initial attempts to determine the structure of the quaternary complex of IL-15 yielded crystals that diffracted to a resolution of only 3.8 Å. To obtain a structure of higher resolution, we did reductive methylation of the complex. This mild chemical modification results in dimethylation of surface lysine residues that can often improve crystal quality among the fifteen residues that contact IL-2Rβ (Fig. 2e). Asp8 forms hydrogen bonds to His133 and Tyr134 of IL-2Rβ; Asp61 forms a salt bridge with Lys71; and Asn65 contacts the triad of Arg42, Gln70 and Tyr134. The importance of these residues for both IL-2 and IL-15 has been confirmed by mutagenesis studies13–16. Of the remaining site I contact residues in IL-15, many are relatively conservative substitutions of those in IL-2 and interact with IL-2Rβ in a similar way. For example, Val91 and Ile92 of IL-2 form van der Waals interactions with Thr73 and Val75 of IL-2Rβ; in IL-15, the same contacts are made by Ile68 and Lys69. However, there are some notable differences in the binding chemistry of the IL-2–IL-2Rβ and IL-15–IL-2Rβ interfaces. IL-2 recognizes Glu136 of IL-2Rβ through a hydrophobic interaction between Leu19 and the aliphatic portion of the glutamic acid side chain (Fig. 2b). In the IL-15 site I interface, this interaction has a completely different character, as Glu136 forms a hydrogen bond to Tyr134.

Figure 1. The crystal structure of the quaternary IL-15 receptor complex. (a) Front view (left) and top view (right) of the IL-15 quaternary receptor complex composed of IL-15 (green), IL-15Rα (cyan), IL-2Rβ (blue) and γc (gold), including the site I and site II interactions of IL-15 with IL-2Rβ and γc, respectively (left). (b) The structure of the IL-2 quaternary complex (Protein Data Bank accession code 2B51; left), and superimposition of the IL-15 and IL-2 receptor complexes (right; r.m.s.d., 1.175 Å).
hydrogen bond with Ser7 of IL-15 (Fig. 2a). Another salient feature of the IL-15–IL-2Rβ interface is the lysine pair Lys10 and Lys11. Lys10 forms a salt bridge with Glu136 of IL-2β that has no equivalent in IL-2, whereas Lys11 seems to satisfy the role of two IL-2 residues at the site I interface. Pointing upward from helix A toward helix C, Lys11, like Met23 of IL-2, presents the aliphatic portion of its side chain for van der Waals interactions with His133 of IL-2β while positioning its terminal amine at the same site as the guanidinium of Arg81 of IL-2 (Fig. 2a–c). Thus, whereas the three key contact residues in the interface are maintained in IL-2 and IL-15, the overall divergence of the IL-2β-binding chemistry suggests that cytokine-specific structural mimicry of site I, the most critical ‘hot-spot’ residue Gln126 of IL-2 is conserved in IL-15 as Gln108 and packs neatly into the same surface. Dark yellow indicates Tyr103 of IL-15 (green cylinders and side chains) in contact with IL-2Rβ (blue loops and side chains). (c) Superimposition of the A and C helices of IL-15 (green) and IL-2 (magenta), showing structural conservation of Asp61, Asn65 and Asp8 of IL-15. (d) Superimposition of IL-2Rβ bound to IL-15 (light blue) and IL-2 (blue), indicating the apparent rigidity of the interface in binding two distinct cytokines.

has some unique features, a similar docking strategy is used for the binding of γc to IL-2 and IL-4, and this emphasizes the cross-reactive properties of the γc cytokine-binding surface, which is able to engage all members of the γc cytokine family. In particular, the absence of highly charged bonds would facilitate degenerate cytokine binding. Like IL-2 and IL-4 (data not shown), IL-15 interacts with the EF1, BC2 and FG2 loops of γc by side chains positioned by the A and D helices (Fig. 3a,b). In a similar example of the three-dimensional structural mimicry of site I, the most critical ‘hot-spot’ residue Gln126 of IL-2 is conserved in IL-15 as Gln108 and packs neatly into the same trench of γc formed by residues Pro207, Cys209, Gly210 and Ser211. Similarly, Tyr103 of γc, which is mutant in some people with X-linked severe combined immunodeficiency and is critical for optimal ligand binding, is recognized by parallel mechanisms in IL-2 and IL-15; the phenyl ring packs with Ser127 of IL-2 and Met109 of IL-15, whereas the hydroxyl moiety makes a hydrogen bond to Ser130 of IL-2 and Asn112 of IL-15.

IL-15 (108 residues) is smaller than IL-2 (133 residues), and a distinct structural feature in site II seems to have evolved to compensate for this difference. In the site II interface of IL-15, there is an additional region of contact between residues on the A-B loop of IL-15 and the CC’1 loop of γc (Fig. 3a,c,d). This interface buries an area of 490 Å², which constitutes over one third of the entire buried surface area of the site II interface of IL-15 (1367 Å²). IL-2 forms a much smaller interface with this region of γc contributing only 70 Å², or 7%, of the 995 Å² of total buried surface area of site II (Fig. 3d). IL-15 has shorter A and D helices than does IL-2 (2 turns and 1.5 turns

Figure 3 Comparison of the site II interfaces of IL-15 and IL-2. (a) The site II interface of IL-15 (green tubes and side chains) bound to γc (gold surface). Dark yellow indicates Tyr103 of γc (residue associated with X-linked severe combined immunodeficiency). (b) The site II interface of IL-2 (magenta tubes and side chains) bound to γc (gold surface). (c) Superimposition of the A and D helices of IL-15 (green) and IL-2 (magenta). Only Gln108 and Ile111 of IL-15 are strictly conserved at the interface. (d) Binding interfaces on the surface of γc, shaded according to binding to IL-15 (left; dark green) or IL-2 (right; magenta).
IL-15Rα presentation has proven to be a major mechanism of IL-15 action from solution, which results in a much lower entropic barrier for the formation of IL-15Rα from those helices. Although IL-2 has ten contact residues located in the cytokine to IL-2Rβ and γc cytokines, as well as the less favorable shape complementarity if IL-15 with γc. IL-15 assembles the IL-2Rβγc signaling complex in a way nearly indistinguishable from that of IL-2, despite their shared and unique molecular recognition strategies in binding the receptor subunits. The great overall similarity of the structures of the IL-15 and IL-2 complexes disfavors structural explanations for the unique functional properties of the cytokines. However, the details of the cytokine and receptor contacts present structural opportunities for the specific disruption or enhancement of either cytokine for therapeutic purposes.

**Molecular insight into IL-15 trans signaling**

Signaling through the receptors for IL-2 and IL-15 is initiated when IL-2Rα or IL-15Rα captures IL-2 or IL-15, respectively, and presents the cytokine to IL-2Rβ and γc. IL-15, however, can signal through an unusual mechanism whereby it is present in trans by cells expressing IL-15 and IL-15Rα to IL-2Rβγc-responsive cells expressing IL-2Rβ and γc (ref. 19). Unlike the situation in cis, IL-15 trans signaling does not benefit from the substantial surface-capture effect of the binding of IL-15Rα to IL-15 on the same cell, as is the case for IL-2. A major role for IL-2Rα is simply to enrich the cell surface by capturing IL-2 and IL-15Rα complex indicated that the IL-15–IL-15Rα complex does not undergo a substantial conformational change after binding IL-2Rβ and γc (ref. 19). We hypothesized that IL-15Rα might thus stabilize a conformation of IL-15 that is more able to bind IL-2Rβ, akin to the effect of IL-2Rα for IL-2. Direct comparison of free and IL-15Rα-bound IL-15 is not possible at present, as the structure of free IL-15 has yet to be elucidated, possibly because of the biochemical instability of the molecule in the absence of IL-15Rα20. We instead turned to computational approaches to investigate the potential structural and dynamics influences of IL-15Rα on IL-15.

Proteins exist in solution as flexible conformational ensembles whose equilibrium can be perturbed after ligand binding. For example, IL-2 has been shown to be very conformationally plastic. Using molecular dynamics simulations, we sought to determine how binding to its α-receptor subunit alters the conformational ensemble of IL-15. We constructed an atomically detailed Markov-state model (MSM) whose equilibrium can be perturbed after ligand binding. For example, IL-2 has been shown to be very conformationally plastic. Using molecular dynamics simulations, we sought to determine how binding to its α-receptor subunit alters the conformational ensemble of IL-15. We constructed an atomically detailed Markov-state model (MSM) whose equilibrium can be perturbed after ligand binding. For example, IL-2 has been shown to be very conformationally plastic.
free-energy landscape that ultimately determines the system's structure and dynamics. Using these MSMs, we calculated the average r.m.s. deviation for each structural element under the two conditions (Fig. 4c). This analysis showed that the conformational freedom of the A-B and C-D loops was greatly restricted in the bound state, as expected, given that these loops form the contacts to IL-15Rα. To a lesser extent, there seemed to be global stabilization of the four helices. Visualization of the most highly populated conformations from each set of conditions showed that the differences were subtle, both in the helices and loops (Fig. 4d). This was in contrast to IL-2, for which binding of IL-2Rα specifically repositions the B and C helices of IL-2 for optimal binding to IL-2Rβ23,24. Despite such disparate mechanisms (global versus helix-specific stabilization), our results suggested that IL-15Rα and IL-2Rα share the property of conformationally stabilizing relatively flexible cytokine ligands to decrease energetic barriers to binding and increase the affinity of IL-15 and IL-2 for IL-2Rβ.

**Comparison of IL-15 and IL-2 signaling properties**

There is considerable controversy about whether IL-2 and IL-15 yield different intracellular signals after activation of the receptor. Although some studies have found that the cytokines produce indistinguishable signaling profiles28, others have demonstrated substantial differences. These differences have been reported to be alterations in signaling kinetics29,30 and efficacy31 for individual pathways. Given the considerable structural similarity of the quaternary complexes of IL-2 and IL-15, we sought to reexamine their membrane-proximal signaling activities. For this, we determined the dose-response relationships and signaling kinetics of IL-2 and IL-15 on cells expressing or deficient in IL-2Rα and IL-15Rα. We took advantage of the human NK cell line YT-1, which we sorted into separate IL-2Rα+ and IL-2Rα− subpopulations (Supplementary Fig. 3a) for dose-response and kinetic analysis of the phosphorylation of the transcription factor STAT5 and kinase Erk, as assayed by flow cytometry with phosphorylation-specific antibodies (Fig. 5). We also isolated CD8+ T cells from mouse spleens and IL-15–IL-15Rα complexes in IL-2Rα+ YT cells (top left) and IL-2Rα− YT cells (top right); signaling kinetics relationships for phosphorylated STAT5 (second row) or Erk1/2 (third row); and internalization kinetics of IL-2Rβ (presented relative to maximal surface expression; bottom row). Horizontal axes of top row indicate the log of cytokine concentration in nM; all kinetics experiments (rows below) used a saturating concentration (500 nM) or subsaturating concentration (1 nM) of each cytokine. MFI, mean fluorescent intensity. Data are representative of at least two experiments per panel (error bars, s.e.m. of triplicates).

EC50 values being IL-15—IL-15Rα = H9 < IL-15 < IL-2 (Fig. 5, top left). The somewhat lower EC50 value of IL-15 than that of IL-2 may have resulted from the small amount of IL-15Rα expressed on YT-1 cells (Supplementary Fig. 3a). When IL-2Rα was present, the EC50 rank order was H9 = IL-2 < IL-15—IL-15Rα < IL-15, which reflected the surface-capture and avidity effects of membrane-bound IL-2Rα on IL-2 and H9 (Fig. 5, top right). We obtained similar results when we compared the dose-response relationships of freshly isolated and preactivated mouse CD8+ cells, but with a few distinctions. On freshly isolated CD8+ cells, free IL-15 produced a biphasic dose-response relationship, consistent with the low expression of IL-15Rα in these cells, including a high proportion of IL-15Rα− cells (Fig. 6, top left, and Supplementary Fig. 3b). Notably, IL-15—IL-15Rα complexes did not demonstrate a biphasic dose-response curve, suggestive of the ability of soluble IL-15Rα to impede engagement of membrane-bound IL-15Rα. The subsequent rank order of EC50 values was IL-15 (EC50 value 1) < H9 < IL-15—IL-15Rα < IL-15 (EC50 value 2) = IL-2. On preactivated CD8+ cells, the curves for the EC50 values of IL-2 and IL-15 shifted substantially to the left (Fig. 6, top right), which reflected the potent effect of the expression of IL-2Rα and IL-15Rα on cytokine sensitivity. The curve for H9 also shifted to the left, as it is able to bind IL-2Rα and benefit from surface capture, but the EC50 value of the IL-15—IL-15Rα complex was essentially unchanged relative to that of freshly isolated cells. For all cells and regardless of differences in EC50 values, IL-2, H9 and the IL-15—IL-15Rα complex stimulated equivalent phosphorylation of STAT5, Erk and S6R at saturating doses.

We next monitored the kinetics of signaling by IL-2 and IL-15 with subsaturating doses (1 nM or 20 pM) and saturating doses (500 nM or 10 nM) of IL-2, H9, IL-15 and IL-15—IL-15Rα complexes. We assayed the three main IL-2 and IL-15 signaling pathways (Jak-STAT, Ras-MAPK and PI(3)K-Akt) and found their signaling kinetics were very much dependent on concentration and α-receptor subunit (Figs. 5 and 6, middle and bottom). In particular, both the rate and magnitude of signaling for each pathway were readily predicted by their respective concentration-response relationships. For example, at subsaturating concentrations and in the absence of IL-2Rα, IL-2 had the slowest signaling kinetics of all the cytokines, which matched its right-shifted dose-response curve under those conditions.
As with intracellular signaling, differences in the gene expression elicited by IL-2 and IL-15 induce similar gene-expression profiles after we accounted for variability in expression of the membrane-proximal signaling, could be explained by concentration-dependent effects, or if the two cytokines produce fundamentally different gene-expression profiles. To maximize our chances of detecting genes regulated differently by IL-2 and IL-15, we used RNA sequencing to compare the gene-expression profiles of CD8+ T cells stimulated with saturating cytokine concentrations commonly used by other investigators in the field (1 nM) or saturating concentrations of each cytokine (500 nM). Two-dimensional multidimensional scaling plot analysis showed that IL-2- and IL-15-regulated mRNA correlated at each time point and concentration (Supplementary Fig. 4a).

Figure 6 Analysis of signaling by IL-2 and IL-15 in primary mouse CD8+ cells. Dose-response relationships (as in Fig. 5) of phosphorylated STAT5 with IL-2, H9, IL-15 and IL-15–IL-15R complexes in freshly isolated CD8+ T cells (top left) and CD8+ T cells preactivated with anti-CD3 (top right), and signaling kinetics relationships for phosphorylated STAT5 (second row) and phosphorylated S6R (bottom row) at a saturating concentration (500 nM or 10 nM) or subsaturating concentration (1 nM or 10 pM) of each cytokine. Data are representative of two experiments (error bars, s.e.m. of duplicates).

(Figs. 5 and 6, far left). We noted a similar trend for the IL-15–IL-15R complexes on preactivated cells at subsaturating conditions (Fig. 6, middle left). In contrast, all four stimuli produced overlapping and nearly identical kinetic profiles for the phosphorylation of STAT5, Erk and S6R at saturating cytokine concentrations (Figs. 5 and 6, middle left and far right). Consistent with the kinetic phosphorylation profiles reported above, downregulation of the signaling receptor IL-2Rβ also demonstrated a strong relationship with cytokine affinity and concentration (Fig. 5, bottom). Specifically, the cytokines with higher affinity drove faster and more complete internalization of IL-2Rβ at lower cytokine concentrations, but the differences were diminished at saturating doses. Together these results indicated that IL-2 and IL-15 generated very similar, if not identical, intracellular signals after we accounted for variability in expression of the α-receptor subunit and cytokine-receptor affinity.

IL-2 and IL-15 induce similar gene-expression profiles

As with intracellular signaling, differences in the gene expression induced by IL-2 and IL-15 have been reported, which perhaps account in part for functional differences between these two cytokines. We wondered if these differences, like the reported differences in membrane-proximal signaling, could be explained by concentration-dependent effects, or if the two cytokines produce fundamentally different gene-expression profiles. To maximize our chances of detecting genes regulated differently by IL-2 and IL-15, we used RNA sequencing to compare the gene-expression profiles of CD8+ T cells stimulated with saturating cytokine concentrations commonly used by other investigators in the field (1 nM) or saturating concentrations of each cytokine (500 nM). Two-dimensional multidimensional scaling plot analysis showed that IL-2- and IL-15-regulated mRNA correlated at each time point and concentration (Supplementary Fig. 4a). As noted for the membrane-proximal signaling induced by IL-2 and IL-15, the gene-expression profiles elicited by these cytokines were more similar when the cytokines were applied at saturating concentrations ($r^2 = 0.909$ and $0.962$ at 4 and 24 h, respectively; Fig. 7a, bottom) than when they were applied at subsaturating concentrations ($r^2 = 0.784$ and 0.611 at 4 and 24 h, respectively; Fig. 7a, top). To identify IL-2- and IL-15-regulated genes, we chose those with over five reads per kilobase of exon model per million mapped reads that also had a change in expression of twofold or more at any time point relative to their expression in unstimulated control cells. This analysis identified 4,690 genes regulated by IL-2 and 4,776 genes regulated by IL-15; many of the same genes were regulated by both cytokines, so a total of 5,182 different genes were regulated by at least one of these cytokines (Supplementary Fig. 4b). There was similar expression of 90.5% of the IL-2-regulated genes and 92.2% of the IL-15-regulated genes after stimulation with IL-2 or IL-15 (difference in expression of less than twofold). In contrast, 406 genes were more potently regulated by IL-2 than by IL-15 (ratio of expression after stimulation with IL-2 to expression after stimulation with IL-15, >2), and 492 genes were regulated more potently by IL-15 than by IL-2 (Fig. 7b–d, Supplementary Fig. 4b and Supplementary Spreadsheets 1 and 2).

Having identified candidate genes that may be regulated differently by IL-2 and IL-15, we sought to confirm the gene-expression differences...
and determine if they persisted independently of concentration. Thus, we stimulated CD8+ T cells with a subsaturating concentration (1 nM) or saturating concentration (500 nM) of each cytokine and assayed by quantitative RT-PCR the expression of a set of genes over a broad range of cytokine concentrations and kinetic intervals and found that many of the apparent signaling differences between IL-2 and IL-15 could be explained by differences between the two cytokines in receptor affinity. Similarly, we found high correlation in the gene-expression profiles of cells stimulated with IL-2 and IL-15 and that differences in gene expression were generally lower at saturating concentrations of the cytokines. When differences persisted at saturation, they remained modest, which brought into question their true biological relevance. Although our results do not rule out the possibility of additional mechanisms of IL-15 action, they indicate that these mechanisms are not necessary to explain the complex and diverse functions of IL-15 and IL-2 observed in vivo. Instead, we found that the expression of the α-receptor subunit and cytokine concentration substantially affected the signaling activity of IL-2 and IL-15 and produced differences in gene expression when the cytokines were at different points on their respective concentration-response curves. Presumably the disparate spatial and temporal expression of the α-receptor subunits, as well as their absolute expression, dynamically regulates the sensitivity of cells for each respective cytokine and their ensuing response to stimulation.

Underscoring the importance of their respective α-receptor subunits in their functions is the notable difference between IL-2 and IL-15 in the way they are presented to effector cells. As IL-15 binds to IL-15Rα with extremely high affinity and IL-15Rα is widely expressed in tissues, IL-15 is believed to exist in the body mainly in a complex with IL-15Rα and is therefore primed for trans presentation to cells that express IL-2Rβ and γc (ref. 6). As mentioned before, soluble complexes of IL-15–IL-15Rα that mimic trans presentation have greater potency than does free IL-15 (refs. 20–22). Through our studies we have elucidated the mechanism underlying this phenomenon; we found that binding of IL-15Rα increased the affinity of IL-15 for IL-2Rβ approximately 150-fold. This affinity increase for
IL-2Rβ subsequently manifested as a left shift in the concentration-response relationship of IL-15 signaling in cells that lacked IL-15Rα. The structural basis for the affinity enhancement of IL-15 for IL-2Rβ seemed to be a consequence of a much greater degree of global stabilization of IL-15 after it bound to IL-15Rα than of IL-2 after it binds to IL-2Rα23,24. From a teleological perspective, the affinity enhancement endowed by IL-15Rα onto the IL-15–IL-2Rβ interaction may serve to compensate for the lack of surface capture in the setting of trans presentation. IL-2 is administered clinically as immunotherapy for the treatment of renal cell carcinoma and metastatic melanoma. However, IL-2 therapy is hampered by dose-limiting toxicity from vascular leakage and the counterproductive activation of regulatory T cells that abrogate antitumor responses. Both of these undesirable side effects are attributable to the activation of cells that express IL-2Rα: pulmonary vascular endothelial cells and IL-2RαCD4+ regulatory T cells13,14. IL-2 variants that bind to IL-2Rβ with high affinity independently of IL-2Rα (super-2 or H9) have greater antitumor efficacy and result in less pulmonary edema than does wild-type IL-2 (ref. 24). Super-2 activates antitumor responses from IL-2Rα+ cells such as naive T cells and NK cells more efficiently, with proportionally less activation of IL-2Rα+ cells such as regulatory T cells and pulmonary endothelial cells, than does wild-type IL-2. The potential use of IL-15 for therapy is hampered by dose-limiting toxicity from vascular leakage and the counterproductive activation of regulatory T cells; 4GS7; GEO: IL-2 and IL-15 RNA sequencing data, GSE40350.

The degree of dependence on the IL-15Rα-receptor subunit inherent on each molecule must be considered. Although IL-2 and IL-15 represent the extreme ends of the spectrum, showing great dependence on their α-receptor subunits for potency, super-2 and RLI seem to have a dependence between that of the two wild-type cytokines, showing little to no 'preference' for cells that express IL-2Rα or IL-15Rα. Super-2 and RLI can be further distinguished by their interactions with IL-2Rα and IL-15Rα. As the IL-15Rα-binding site is sterically obscured in RLI, it represents the exact midpoint between IL-2 and IL-15 on the spectrum, unaffected by the presence or absence of either α-receptor subunit. In contrast, super-2 is able to bind to IL-2Rα and consequently shows some 'preference' for IL-2Rα+ cells rather than IL-2Rα− cells, albeit to a much lower degree than does wild-type IL-2. This subtle distinction may yield notable differences in efficacy and toxicity. For example, though IL-2-Rα is responsible for many of the undesirable side effects of IL-2, some IL-2Rα+ cells (such as activated T cells) may be beneficial to target. Similarly, IL-15Rα+ cells (such as NK cells and cytotoxic CD8+ cells) are critical determinants of antitumor efficacy in vivo. Given the considerations noted above, it may be possible to enhance immunotherapy with IL-2 and/or IL-15 by modulating their dependence on IL-2Rα and IL-15Rα, respectively, thus ‘tuning’ the distribution of cells of the immune system activated for therapeutic effect. In this context, super-2 and RLI represent good starting points for such immunological manipulation. Just as the structure of the IL-2 quaternary complex enabled the engineering of super-2, we hope to leverage the information obtained from the IL-15 quaternary complex presented here for the design of improved IL-15 therapies.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank: atomic coordinates and structure factors, 4GS7; GEO: IL-2 and IL-15 RNA sequencing data, GSE40350.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.M.R., D.F. and E.O. did crystallographic studies of the IL-15 quaternary complex; A.M.R. and E.O. determined and refined that structure; M.R. did SPR measurements; G.R.B. and V.S.P. did and analyzed molecular dynamics simulations; A.M.R. prepared cytokine proteins for signaling and transcriptional studies; A.M.R., S.M., L.M. and R.S. did signaling experiments by flow cytometry with phosphorylation-specific antibodies; A.M.R. did receptor-internalization studies; J.-X.L. and P.L. did and analyzed RNA sequencing transcriptional assays; J.-X.L. confirmed the quantitative PCR; A.M.R., J.-X.L., G.R.B., W.J.L. and K.C.G. designed the experiments; A.M.R., J.-X.L., P.L., S.M. and G.R.B. prepared the figures; A.M.R., W.J.L. and K.C.G. wrote the paper; and W.J.L. and K.C.G. supervised the research.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Protein expression and purification. For crystallization, the sequences encoding six-histidine-tagged human IL-15 (amino acids 1–114) in pET22b and six-histidine-tagged human IL-15Rα (amino acids 1–67) in pET26b were expressed together in the periplasm of BL21(DE3) Escherichia coli cells by induction for 20 h at 22 °C with isopropyl β-D-thiogalactopyranoside. The periplasmic fraction was isolated by osmotic shock and recombinant protein was purified by nickel–nitrilotriacetic acid chromatography followed by size-exclusion chromatography with a Superdex-75 column into HEPESS-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl and 0.02% sodium azide). Human IL-2Rβ (amino acids 1–214) with substitution of glutamine for asparagine at positions 3, 17 and 45 and human γc (amino acids 34–232) with substitution of glutamine for asparagine at position 53 were expressed and purified from Hi5 insect cells as described21. Purified, E. coli-derived IL-15–IL-15Rα was then mixed with purified, insect-derived IL-2Rβ and γc at a ratio of 1:1:1, followed by treatment with carboxypeptidases A and B overnight at 4 °C. The digested proteins were then methylation as described21 and were purified by size-exclusion chromatography with a Superdex-200 column into HEPESS-buffered saline.

For signaling and SPR experiments, IL-15 (amino acids 1–114) and IL-15 expressed together with IL-15Rα (amino acids 1–64) were produced in Hi5 cells and were purified by nickel–nitrilotriacetic acid and size exclusion chromatography. Biotinylated IL-2Rβ was obtained by the addition of a carboxy-terminal biotin acceptor peptide tag (GLNDIFEAQKIEWHE) and coexpression with the addition of BirA ligase with excess biotin (100 μM) to the expression medium. Human IL-2 (1–133) and its high-affinity variant and coexpression with the addition of BirA ligase with excess biotin (100 μM) of a carboxy-terminal biotin acceptor peptide tag (GLNDIFEAQKIEWHE) were isolated by osmotic shock and recombinant protein was purified by nickel–nitrilotriacetic acid chromatography followed by size-exclusion chromatography with a Superdex-75 column into HEPESS-buffered saline.

Crystallization and data collection. The purified, carboxypeptidase-treated and methylated IL-15–IL-15Rα–IL-2Rβ–γc quaternary complex was concentrated to 12.1 mg/ml and crystallized by vapor diffusion in hanging-drop by addition of 0.1 μL crystallization solution (22.5% PEG3350, 0.1 M Bis-Tris propane, pH 8.75, and 0.2 M sodium acetate) to a volume of 0.1 μl protein. Crystals grew to a maximum size of 150 × 50 × 50 μm after 2–3 d at 22 °C. Crystals were cryo-protected in crystallization solution supplemented with 15% ethylene glycol and were flash-frozen in liquid nitrogen. A data set at 2.35 Å was collected at beamline 8-2 at the Advanced Light Source and was processed with the HKL-3000 system for the integration of data reduction and structure solution36.

Structure determination and refinement. The IL-15 quaternary complex structure was solved by molecular replacement with individual IL-2Rβ and γc subunits from Protein Data Bank accession code 2BS3 and the IL-15–IL-15Rα complex from Protein Data Bank accession code 223C. The Phenix software suite for the automated determination of macromolecular structures37 was used for structural refinement and the COOT (Crystallographic Object-Oriented Toolkit) program for macromolecular model building, completion and confirmation38 was used for model adjustment. Bulk solvent flattening was used for solvent correction. For the initial refinement, rigid body, coordinate and real-space refinement were used with individual atomic displacement parameter refinement. Translation, libration and screw-rotation refinement was added in later refinement iterations.

Buried surface area was calculated with the PISA server (protein interfaces, surfaces and assemblies)39.

Simulations and MSM. The Gromacs 4.5.2 package40 was used for molecular dynamics simulations with the Amber03 force field41. Each structure was placed in a dodecahedral box of about 6.6 × 6.6 × 4.7 nm and solvated with approximately 6,250 ‘transferable intermolecular potential, three-position model’ water molecules. Conformations were first minimized with a steepest descent algorithm with a tolerance of 1,000 kcal/mol/nm and a step size of 0.01 nm. A cutoff of 1 nm was used for Coulombic and Van der Waals interactions and a grid-based neighbor list. Conformations were then equilibrated at 300K and 1 bar by holding protein atoms fixed and allowing the surrounding water to relax for 500 ps with a time step of 2 fs. All bonds were constrained with the LINCS algorithm42. Center-of-mass motion was removed at every step and a grid-based neighbor list with a cutoff of 1.5 nm was updated every 10 steps. For electrostatics, we used the fourth-order particle-mesh Ewald method43 with a cutoff of 1.5 nm for Coulombic interactions, a Fourier spacing of 0.08 nm and a tolerance of 1 × 10−5. A hard cutoff of 1.2 nm was used for Van der Waals interactions with a switch starting at 1 nm. The temperature was controlled with the v-rescale thermostat44 applied to both the protein and solvent with a time constant of 0.5 ps. The pressure was controlled with an isotropic Berenson barostat45 applied to the entire system with a time constant of 0.5 ps and a compressibility of 4.5 × 10−5 bar−1. Long-range corrections were applied to energy and pressure. Production simulations up to 65 ns in length used the same parameters as for equilibration, with the exception that the protein atoms were no longer held fixed.

We used the MSMBuilder software package46 to construct an MSM with a lag time of 1 ns. On the basis of published work on protein folding47,48, we chose to create 208 clusters (microstates) with a hybrid k-centers–k-medoids algorithm and the r.m.s. deviation between pairs of conformations. All Cα and Cβ atoms were used for the r.m.s. deviation. Thermodynamic and kinetic properties were extracted from the eigenvalues and eigenvectors of MSM47,48.

Assignment of residues to structural units used in r.m.s. deviation plots is provided in Supplementary Table 1; as with MSM, all Cα and Cβ atoms were used for these r.m.s. deviations.

SPR. A Biacore T100 was used for SPR at 25 °C. Protein concentrations were quantified by ultraviolet spectroscopy at 280 nm with a Nanodrop2000 spectrometer (Thermo Scientific). Experiments were done on a Biacore SA sensor chip (GE Healthcare), which was used to capture biotinylated IL-2Rβ (Rmax = 90 RU) and γc at a ratio of 1:1:1, followed by treatment with carboxypeptidases A and B overnight at 4 °C. The digested proteins were then purified with size-exclusion chromatography with a Superdex-200 column into HEPESS-buffered saline.

Cell lines. YT-1 cells were maintained in complete RPMI-1640 medium (Gibco) in a humidified incubator at 37 °C and 5% CO2. IL-2Rα+ YT-1 cells were obtained by enrichment with magnetic sorting by phycocerythrin-conjugated anti-IL-2Rα (BC96; Biolegend) and paramagnetic microbeads coated with anti-phycocerythrin (Miltenyi Biotec). Enrichment for IL-2Rα+ cells was assessed by flow cytometry in the FL2 channel with an Accuri C6 flow cytometer.

Analysis of intracellular signaling via STAT5 and Erk1-2 by flow cytometry with phosphorylation-specific antibodies. For dose–response experiments, serial dilutions of IL-15, IL-15–IL-15Rα, IL-2 or H9 were applied to IL-2Rα or IL-2Rα+ YT-1 cells in a 96-well plate (2 × 104 cells per well). After 10 min, cells were fixed in paraformaldehyde and permeabilized in 100% methanol. For analysis of signaling kinetics, 1 or 500 nM IL-15–IL-15Rα, IL-2 or H9 was applied to YT-1 cells (2 × 104 cells per well) and were fixed after 1, 2.5, 5, 15, 30, 60 or 120 min) and were permeabilized with 100% methanol. Samples in methanol were `multiplexed’ (that is, many samples were combined into one tube before staining) through the use of ‘fluorescent barcoding’ (a means by which each sample is labeled with a different signature)49 with amine-reactive DyLight 800 dye (Thermo Scientific) and Pacific blue dye (Invitrogen), then were stained with Alexa Fluor 647–conjugated anti-STAT5 P1Y694 (562076; BD Biosciences) and Alexa Fluor 488–conjugated anti-Erk1–2 P2T02/P2Y04 (43445; Cell Signaling Technology). Mean cell fluorescence was determined with a LSR II (BD). Dose–response and kinetic curves and EC50 values were calculated with GraphPad Prism.

Isolation of CD8+ cells and analysis of intracellular signaling via STAT5 and S6 by flow cytometry with phosphorylation-specific antibodies. Mice CD8+ T cells were isolated from spleens and lymph nodes of C57BL/6 mice by negative enrichment for CD8+ T cells (CD8α+ T cell isolation kit II; Miltenyi Biotec). For cytokine-stimulation assays with freshly isolated cells, cells were
used immediately. For the generation of in vitro preactivated CD8+ T cells, six-well plates were precoated with 2 μg/ml of plate-bound monoclonal anti-CD3 (2C11; produced in-house). CD8+ cells were seeded at a density of 1 × 10^6 cells per ml with 1 μg/ml of soluble monoclonal anti-CD28 (37.31; BD). Cells were cultured for 2 d with stimulation of the T cell antigen receptor, followed by 6 h of rest in fresh culture medium.

For signaling-kinetics experiments with freshly isolated CD8+ T cells, 1 or 500 nM of IL-15, IL-15–IL-15Rα, IL-2 or H9 was applied to 2 × 10^6 CD8+ T cells per well. For signaling-kinetics experiments with preactivated CD8+ T cells, 10 pM or 10 nM of IL-15, IL-15–IL-15Rα, IL-2 or H9 was used for stimulation. CD8+ T cells were fixed immediately after cytokine stimulation with PhosFlow Lyse/Fix buffer (BD) and then were permeabilized with PhosFlow Perm Buffer III (BD). Cells were then stained at room temperature for 30 min in the dark with phycoerythrin-conjugated antibody to STAT5 phosphorylated at Tyr694 (BD Biosciences) and Alexa Fluor 647–conjugated antibody to S6R phosphorylated at Ser235 and Ser236 (D57.2.2E; Cell Signaling Technology). Data were acquired on a FACSCanto (BD Biosciences) and analyzed with FlowJo (Tree Star).

Receptor-internalization experiments. IL-15, IL-15–IL-15Rα, IL-2 or H9 (500 nM) was applied to 2 × 10^6 YT-1 cells in a 96-well plate for 1, 2.5, 5, 15, 30, 60 or 90 min, after which cells were immediately transferred to ice to prevent further receptor internalization. Cells were washed twice with ice-cold FACS buffer (0.5% BSA and 0.5 mM EDTA in PBS) and then were stained for 30 min on ice with allophycocyanin-conjugated anti-human IL-2Rβ (TUGh4; Biolegend) diluted 1:50. Cells were washed twice more with ice-cold FACS buffer and then were fixed for 10 min at room temperature with 1.5% paraformaldehyde in PBS. After fixation, mean cell fluorescence was determined with an Accuri C6 flow cytometer.

RNA sequencing. Splenic CD8+ T cells were isolated from 6-week-old female C57BL/6 mice and treated with 1 nM or 500 nM of IL-2 or IL-15 for the appropriate time, and total RNA was isolated. Three samples treated identically were pooled, then cDNA was synthesized with 2.5 ng of the pooled RNA and TaqMan 2× PCR Master mix (ABI/Ambion) followed by nine cycles with AUP1* and AUP2* primers (Supplementary Table 2) as described.50 After fragmentation with a Bioruptor (Diagenode), fragments 220–400 base pairs in length were isolated with 2% E-Gel (Invitrogen), then ends were repaired and adaptor (Illumina) was added with T4 DNA ligase (New England Biolabs), followed by amplification in seventeen cycles with PE 1.0 and PE 2.0 primers (Illumina) and Phusion High Fidelity PCR Master Mix (New England Biolabs). PCR products were ‘barcoded’ (indexed) and sequenced on an Illumina HiSeq 2000 platform.

RNA sequencing data analysis. Sequenced reads (single-end 36 base pairs) were aligned to the RefSeq mouse gene database (mm8 revision) with the ELAND pipeline. Raw reads that fell on exons of each gene were counted and normalized per kilobase of exon model per million mapped reads were calculated for each gene. The statistical packages in software of the R project for statistical computing were used for multidimensional scaling, linear-regression modeling and analysis of differences in gene expression.

Gene-expression analysis by real-time RT-PCR. First, cDNA was synthesized with 200 ng total RNA, oligo(dT) and Omniscript RT kit (Qiagen), then RT-PCR was done with an ABI 7900 HD Sequence Detection System (primers, Supplementary Table 2) and TaqMan 2× PCR Master mix (ABI/Ambion). Relative expression was calculated based on the cycle number for the control gene RplP7, as its expression was constant under the experimental conditions.

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