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Elucidation of the Interleukin-15 Binding Site on Its Alpha Receptor by NMR

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ABSTRACT: The cytokine interleukin-15 (IL-15) signals through the formation of a quaternary receptor complex composed of an IL-15-specific α receptor, together with β and γ_c receptors that are shared with interleukin-2 (IL-2). The initiating step in the formation of this signaling complex is the interaction between IL-15 and IL-15Rα, which is a single sushi domain bearing strong structural homology to one of the two sushi domains of IL-2Rα. The crystal structure of the IL2-Rα/IL-2 complex has been determined, however little is known about the analogous IL-15Rα/IL-15 binding interaction. Here we show that recombinant IL-15 can be overexpressed as a stable complex in the presence of its high affinity receptor, IL-15Rα. We find that this complex is 10-fold more active than IL-15 alone in stimulating proliferation and survival of memory phenotype CD8 T cells. To probe the ligand/receptor interface, we used solution NMR to map chemical shifts on ^{15}N-labeled IL-15Rα in complex with unlabeled IL-15. Our results predict that the binding surface on IL-15Rα involves strands C and D, similar to IL-2Rα. The interface, as predicted here, leaves open the possibility of trans-presentation of IL-15 by IL-15Rα on an opposing cell.

Interleukin-15 (IL-15[1]) is a four-helix bundle cytokine that was first identified by virtue of its similar function to interleukin-2 (IL-2) as a stimulator of T cell proliferation and cytotoxic lymphocyte activity (*1*−*3*). IL-15 has since been shown to have distinct functions in NK cell development (*4*−*6*), CD8$^+$ memory T cell homeostasis (*7*−*11*), and innate immune responses (*12*).

With the discovery of IL-15, it was shown to share receptors IL-2Rβ (CD122) and common γ chain (γ_c) with IL-2 for cell signaling (*13*). In addition, each cytokine utilizes a unique α receptor, IL-15Rα and IL-2Rα (CD25), to assemble a quaternary signaling complex on the cell surface (*14*, *15*). The α receptors of IL-15 and IL-2 are composed of globular sushi domain(s) "heads", which comprise the cytokine binding site, followed by a Pro/ Thr rich stalk region leading to the membrane, and a short intracellular segment that is not believed to signal (*15*, *16*). Sushi domains are small 2-on-3 β-sandwich protein folds containing two to three disulfide bonds, originally identified in complement factors (*17*). IL-2Rα has two sushi domains (D1 and D2), both of which make contributions to the binding of IL-2 due to a domain swap in which the "top" β strands in each module exchange, resulting in an IL-2 binding surface that is composed of β strands from both domains (*18*). Interestingly, IL-15Rα has only one sushi domain, analogous to the D1 of IL-2Rα, and it is necessary and sufficient for cytokine binding (*19*, *20*). An important difference between IL-15Rα and IL-2Rα is their affinity for the respective cytokine ligand: IL-15Rα binds IL-15 with high affinity (K_d = 100 pM) (*20*), whereas the interaction of IL-2Rα and IL-2 is lower in affinity (K_d = 10 nM) (*21*).

The α receptors have distinct roles in the assembly of IL-15 or IL-2 with the shared receptors on the cell surface. Dubois et al. demonstrated that IL-15 binds receptors IL-2Rβ and γ_c on a cell when cross presented "in trans" by IL-15Rα on a neighboring cell (*22*). This mechanism of trans-presentation occurs in CD8$^+$ memory T cells (*23*) and NK cells (*24*), and requires that both IL-15 and IL-15Rα are expressed by the same presenting cell (*25*). IL-2 signals in an autocrine manner with two proposed mechanisms of assembly based on thermodynamic analyses: secreted IL-2 either binds to IL-2Rα to initiate quaternary complex formation on the same cell or binds to preformed IL-2Rα/IL-2Rβ complexes prior to γ_c association (*21*). Upon quaternary complex formation, both IL-15 and IL-2 activate the JAK1/JAK3 and STAT3/STAT5 signaling cascade (*26*).

Comparison of the IL-15 and IL-2 systems raises important questions. How is specificity for the shared signaling receptors achieved and explained on a molecular level? In the IL-15 system, can the trans-presentation mechanism be supported structurally? One approach is to look at the structural differences in the cytokine/receptor interactions,

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[1] Abbreviations: IL-15, interleukin-15; IL-2, interleukin-2; γ_c, common γ chain; MP, memory phenotype.

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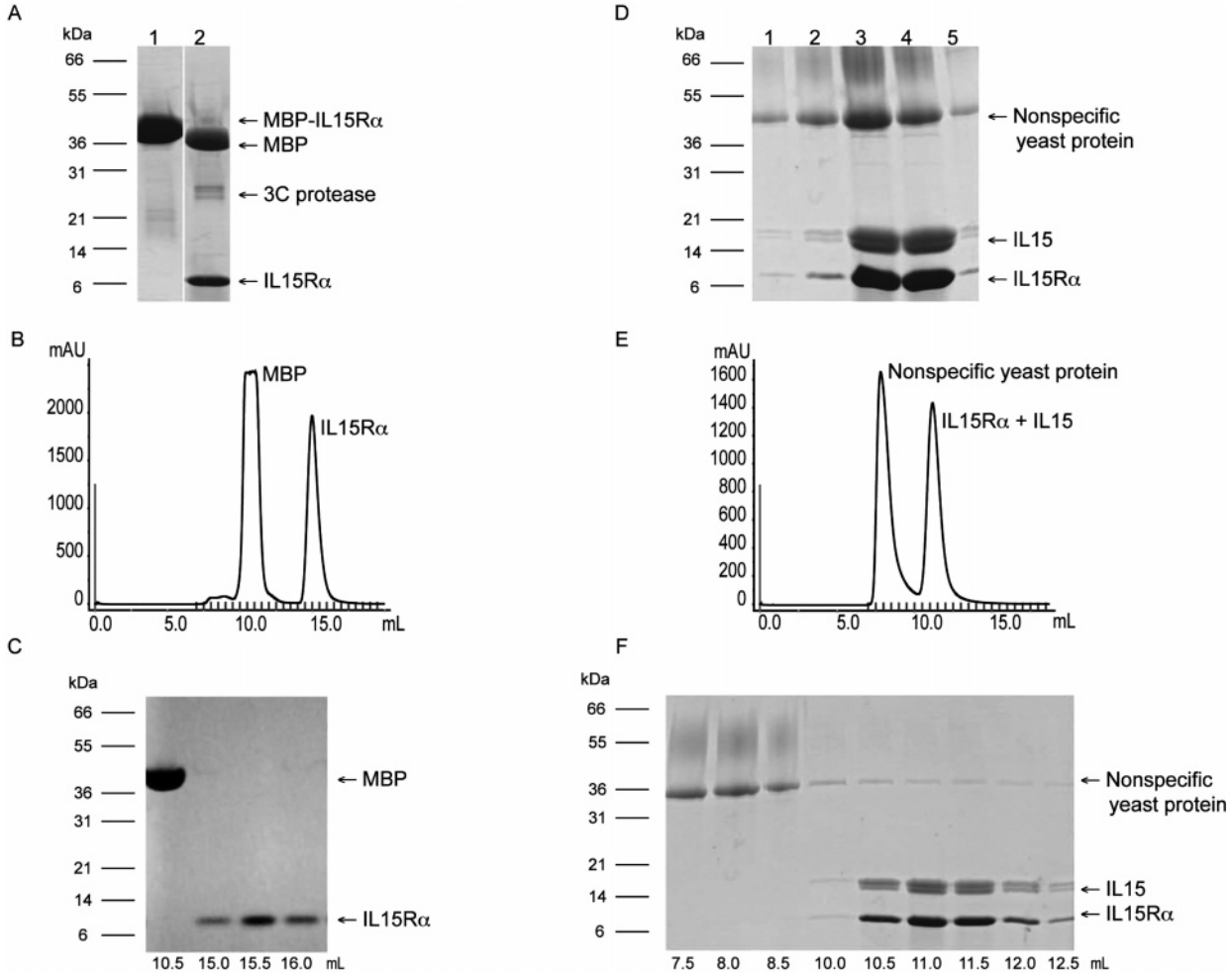

FIGURE 1: Expression and purification of IL-15Rα and IL-15. IL-15Rα was expressed in *E. coli* as an MBP fusion for secretion to the periplasm and was purified using a Ni-NTA column (A, lane 1). IL-15Rα-MBP was digested with 3C protease, and an amylose column was used to remove a portion of the free MBP (A, lane 2). IL-15Rα was then isolated by gel filtration (B, C). IL-15 was expressed in yeast with ^{15}N-labeled IL-15Rα present at induction. The complex was subjected to Ni-NTA column purification (D), and fractions 3 and 4 were combined and purified by gel filtration (E, F).

and in particular the impact of the α receptors on recognition. The quaternary structure of IL-2 was determined by crystallography (*18*), and the solution structure of IL-15Rα was recently determined by NMR (*27*). However, how the high affinity IL-15Rα receptor interacts with IL-15 and how this interaction is structurally related to the IL-2Rα/IL-2 complex remain unknown.

To address this question, we have taken two approaches: First, we have tested the potency of T cell activation comparing both IL-15 and IL-15/Rα complex. Second, we have performed NMR chemical shift mapping on the sushi domain of IL-15Rα in complex with IL-15, and compared the binding interface to that of IL-2Rα and IL-2. NMR chemical shift mapping is a qualitative experiment that identifies residues with different chemical environments between a free state and in complex with a binding partner. Residues with chemical shift differences can be the result of direct interactions at the interface of the cocomplex, or due to indirect conformational changes.

Here we find that the soluble IL-15/Rα complex is more potent than IL-15 alone in T cell simulation. Second, we show that IL-15Rα contains residues with different chemical shifts in the free and cocomplex forms along strands C and D of the receptor, and to a lesser extent strand E, likely forming the interface with IL-15. Chemical shift differences distal to the predicted binding site suggest that cytokine-induced structural accommodations in the receptor may occur upon cocomplex formation. Importantly, this predicted interface allows for the trans-presentation paradigm, as the face of IL-15Rα that binds IL-15 could easily face away from the flexible stalk of the receptor and the presenting cell membrane.

EXPERIMENTAL PROCEDURES

Expression and Purification of IL-15Rα. The sushi domain of IL-15Rα (residues 31−96) was subcloned into the pMAL p2x vector (New England Biolabs) containing a maltose binding protein (MBP) fusion, 3C Rhinovirus protease site at the N terminus, and a His tag at the C terminus, using restriction sites *Nco*I and *Hin*dIII.

The MBP-IL-15Rα fusion protein was secreted into the periplasm of the *Escherichia coli* strain BL21 (*28*). Expression was induced with 1 mM ITG in minimal M9 media (11 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 40 mg/L thiamine, 50 mg/L ampicillin, 0.12 g/L MgSO4, 0.029 g/L $CaCl_2$, 0.5 g/L ^{15}N NH_4Cl, 2 g/L glucose). Protein was extracted by an osmotic shock procedure (*28*), dialyzed, and purified by Ni-NTA affinity chromatography. IL-15Rα-MBP

was digested with 3C Rhinovirus protease (50:1) at 4 °C. The free MBP was removed over an amylose resin column (New England Biolabs), followed by size exclusion chromatography using a Superdex 75 column (Tricorn). IL-15Rα fractions were pooled, and the final NMR sample was prepared by buffer exchange into 20 mM $NaPO_4$, 150 mM NaCl, 0.03% NaN_3, 7% D_2O, pH 7.4.

Expression and Purification of IL-15. The open reading frame of human IL-15 (1−114) was subcloned into pRSGAL (*29*), a yeast secretion plasmid with a GAL promoter, a N-terminal synthetic PrePro sequence for targeting to the endoplasmic reticulum, a N-terminal FLAG epitope tag (YKDDDDK), and a modified C-terminal EYMPME epitope tag. The three potential N-linked glycosylation sites in IL15 (N71, N79, and N112) were mutated to glutamine to prevent potential glycosylation, in order to avoid the frequent hyperglycosylation seen in yeast cells, and achieve the most homogeneous protein possible. The plasmid was transformed by electroporation into the *Saccharomyces cerevisiae* strain YVH10, modified from the yeast strain BJα5464 to overexpress protein disulfide isomerase under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (*29*, *30*). The transformants were grown at 30 °C for 40 h in synthetic selective medium SD-SCAA (−ura, −leu, +trp) containing 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, 5.4 g/L Na_2HPO_4, 8.6 g/L $NaH_2PO_4 \cdot H_2O$, and 3.9 g/L synthetic amino acids. To induce protein expression, cells were pelleted by centrifugation and resuspended in synthetic selective medium containing galactose in place of dextrose. Purified IL-15Rα receptor (0.5 mg/L) was added to the yeast cultures to form a stable ligand/receptor complex upon IL-15 secretion. Bovine serum albumin (0.5 mg/L) was added as a carrier protein to prevent secreted IL-15 from nonspecifically adhering to flask walls. Cultures were grown under inducing conditions at 20 °C for 72 h.

After induction, yeast cultures were harvested by centrifugation. The supernatant containing IL-15Rα/IL-15 was clarified by filtration (0.2 μm), and was concentrated by tangential flow filtration (PALL) using a 10 kDa Centromate membrane. The IL-15Rα/IL-15 complex was purified by Ni-NTA affinity chromatography, followed by size exclusion chromatography using a Superdex 75 column (Tricorn). Fractions were pooled and buffer was exchanged into the final NMR buffer (as above).

Cell Culture and FACS Analysis. Memory-phenotype (MP) $CD8^+$ cells were purified from lymph nodes of IL-7 transgenic mice, which are predominantly $CD44^{hi}$ $CD122^{hi}$ phenotype (*31*). Cell purification and CFSE (Molecular Probes)-labeling was performed using previously described methods (*32*). MP $CD8^+$ cells were cultured in 96-well microtiter plates for 4 days with free IL-15 (R&D Systems) or IL-15/Rα complexes and the cultured cells were stained with PE conjugated anti-CD44 (eBioscience) and Cy5-conjugated anti-CD8 (eBioscience) and propidium iodide (Sigma) and analyzed by a flow cytometer as described (*32*).

NMR. HSQC experiments were acquired for ^{15}N IL-15Rα alone and in complex with unlabeled IL-15. All experiments were run at 25 °C on Varian Inova 600 or 800 MHz spectrometers, processed with NMRPipe (*33*) and analyzed with Sparky software (*34*). To determine the significance of individual chemical shifts, the difference between proton and nitrogen shifts was measured using Sparky software, and chemical shift changes were scored as significant if greater than 0.05 ppm in the proton dimension and/or 0.2 ppm in the nitrogen dimension.

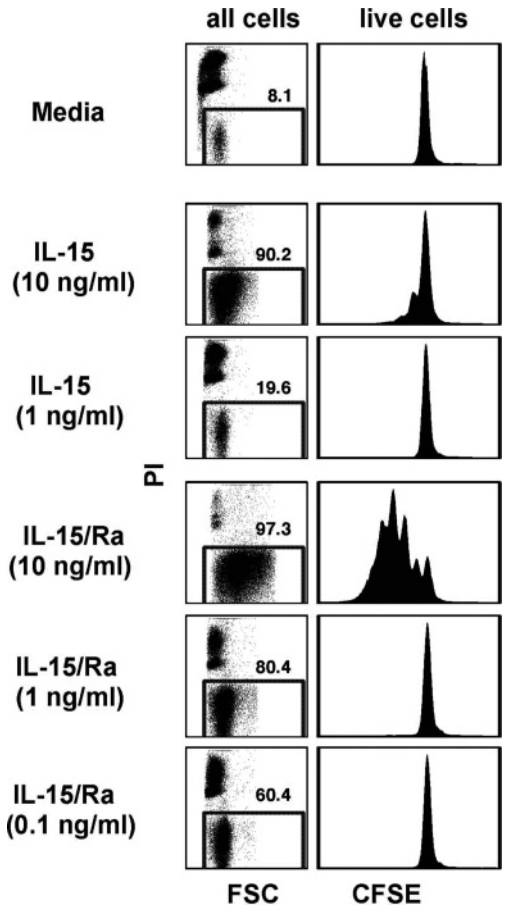

FIGURE 2: IL-15/Rα complexes display significantly higher biological activity than free IL-15. Small numbers (10^5 cells/well) of CFSE-labeled memory phenotype (MP) $CD8^+$ cells were cultured with indicated titrating doses of IL-15/Rα complexes or free IL-15 for 4 days and analyzed after staining for CD8, CD44, and propidium iodide (PI). Shown are the percentages of PI^- live cells (left column) and the CFSE profiles on live cells (right column). MP cells were >98% $CD8^+$ and ∼90% $CD44^{hi}$. One other experiment showed the same results.

Model of IL-15. Based on a sequence alignment of IL-15 and IL-2 (*35*), modeling of IL-15 was performed with Modeller version 8v2 (*36*) using the crystal structure of IL-2 (PDB ID: 1Z92 (*37*)) as a template. SSM Superposition (*38*) was implemented in Coot (*39*) to align the NMR structure of IL-15Rα (PDB ID: 2ERS (*27*)) onto IL-2Rα, and the model of IL-15 onto IL-2. The structural figures were produced with Pymol (*40*).

RESULTS

Protein Expression and Purification. Production of large amounts of recombinant IL-15 has been a recalcitrant problem for studying this system. While small amounts of IL-15 can be produced in yeast (*41*), we and others have found that free IL-15 is highly prone to aggregation. Biologically this may be rationalized by the extremely high affinity of IL-15 for IL-15Rα (K_d = 100 pM), which suggests that *in vivo* IL-15 primarily exists as a complex with IL-15Rα. We tested commercially produced IL-15 from *E. coli* for IL-15Rα binding by native gel, and found that while

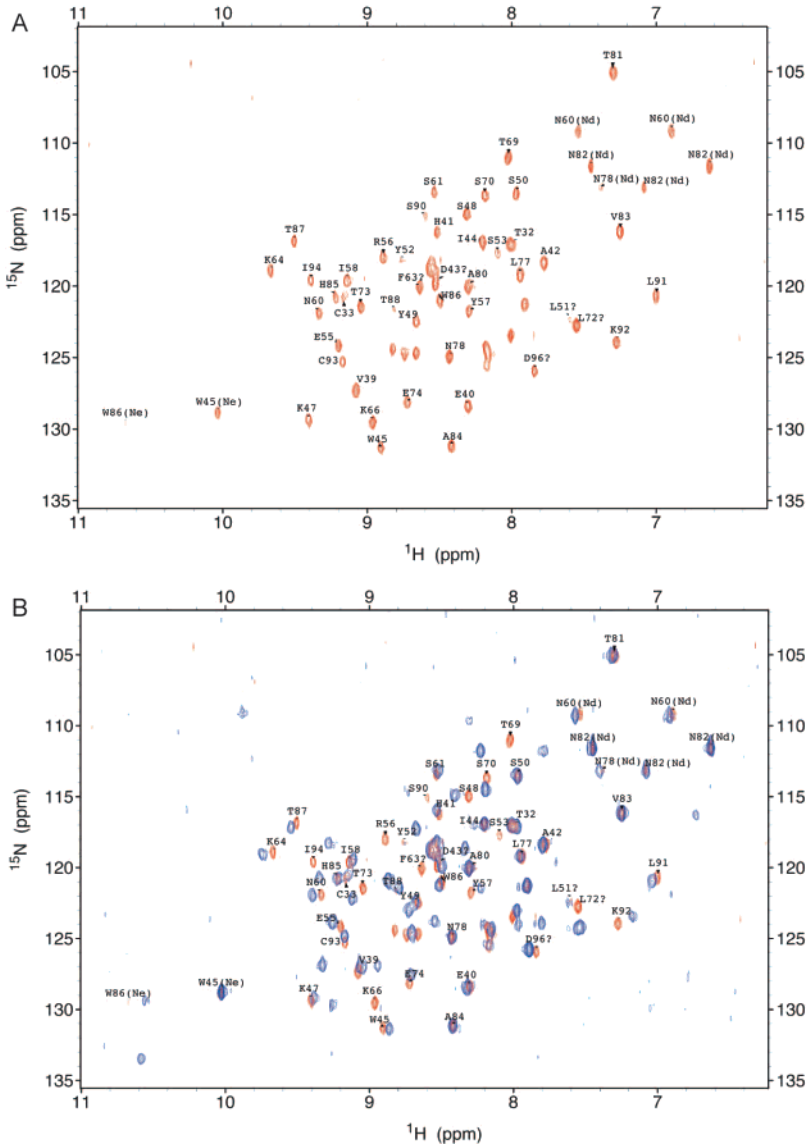

FIGURE 3: NMR of free IL-15Rα and in complex with IL-15. ^{1}H−^{15}N HSQC spectra of the sushi domain of free IL-15Rα (A, red) and superposition of its complex with unlabeled IL-15 (B, blue).

~50% of the material bound IL-15Rα, a significant proportion did not enter the gel and was presumably aggregated. Free IL-15 at the extremely low biologically relevant concentrations may not suffer from such biochemical problems. Nevertheless, we were able to overcome this problem and achieve high-level expression of IL-15 in yeast by addition of its high affinity α receptor during expression, in effect mimicking the expression of IL-15 by a cell bearing IL-15Rα on its surface. We used a version of IL-15 in which we mutated the three asparagine-linked glycosylation sites to glutamine in order to avoid the production of high-molecular-weight hyperglycosylated material as is often seen in yeast, and express the most homogeneous protein possible. These three N-linked sites map far away from the IL-15Rα binding region of IL-15. Glycosylation is not required for biological activity, and the non-N-linked IL-15 behaves in all respects similarly to glycosylated IL-15, which we have produced from insect cells. We cannot rule out the possibility of some O-linked glycosylation on the yeast-expressed material.

We first produced the free α receptor, sushi domain of IL-15Rα (7 kDa), in *E. coli* with an N-terminal MBP tag (Figure 1A, lane 1). The IL-15Rα receptor was cleaved from the MBP tag with 3C protease and purified with an amylose resin column as a first round of removing free MBP (Figure 1A, lane 2). IL-15Rα was isolated by gel filtration (Figure 1B,C) and remained stable in solution.

Purified IL-15Rα was added to yeast cultures at the time of induction to stabilize IL-15 in the media following secretion. The high affinity of IL-15 for IL-15Rα allowed the cocomplex to be purified from yeast supernatant by Ni-NTA chromatography (Figure 1D). Adding the receptor during expression of IL-15 was critical as IL-15 alone, both glycosylated and nonglycosylated, was highly unstable when expressed from yeast, *E. coli*, or baculovirus. We observed a double band of IL-15 on SDS−PAGE gels, which may be due to O-glycosylation of serine or threonine residues, since the material contains no N-linked glycosylation, and material expressed from insect cells also shows this doublet. The exact source of the heterogeneity is unclear. However, both bands of the doublet bind IL-15Rα indistinguishably. Gel filtration chromatography was used to remove yeast proteins which nonspecifically bound to the Ni-NTA resin, resulting in a single peak corresponding to the IL-15Rα/IL-15 complex

(Figure 1E,F). This material was monodispersed and soluble at extremely high concentrations (>30 mg/mL).

Biological Activity of the IL-15Rα/IL-15 Complexes. To determine the relative biological activity of IL-15Rα/IL-15 complexes, CFSE-labeled purified memory-phenotype (MP) $CD8^+$ cells were cultured with titrating doses of IL-15/Rα complexes or control free IL-15. Analysis of proliferation and survival of MP $CD8^+$ cells 4 days later showed that IL-15/Rα complexes displayed significantly better activity than free IL-15 (Figure 2). Thus, while a dose of 10 ng/mL of free IL-15 is very weakly mitogenic for MP $CD8^+$ cells, this dose of IL-15/Rα complexes induced efficient proliferation of MP $CD8^+$ cells. Similarly, IL-15/Rα complexes were at least 10-fold better than free IL-15 in maintaining survival of MP $CD8^+$ cells. This result is in accord with recent work describing the enhanced potency of IL-15 when administered with either soluble IL-15Rα sushi domain (*20*) or IL-15Rα-Fc in stimulating MP CD8 T cells and NK cells (*42*, *43*). However, as mentioned, the exact quantification of the activity of the free IL-15 is subject to some ambiguity due to the presence of some inactive free IL-15 in the sample.

NMR Chemical Shift Mapping of the IL-15Rα/IL-15 Complex. The solution structure of IL-15Rα was recently determined by NMR (*27*), however the nature of the interaction with IL-15 remains unknown. To address this question, we performed NMR chemical shift mapping of IL-15Rα with and without IL-15 to probe changes that occur in the spectrum of the receptor upon ligand binding. The free ^{15}N-labeled IL-15Rα receptor was concentrated to 0.1 mM, and an HSQC spectrum was acquired at 25 °C (Figure 3A). Our spectrum reproduces the footprint of the IL-15Rα sushi domain spectrum as previously published, allowing us to confirm 46 out of 56 backbone amide resonance peaks, and 8 out of 8 side chain peaks with previous assignments (*27*). Of the remaining ten residues from the 66-residue receptor, six were not visible in our HSQC experiments, similar to the previously published spectra, likely due to chemical exchange at pH 7.4 (*27*), and four residues are proline, which do not produce a backbone amide resonance.

Next we produced a sample of ^{15}N-labeled IL-15Rα and unlabeled IL-15 complex at a concentration of 0.1 mM and repeated the HSQC experiment (Figure 3B). To simplify data analysis, IL-15 was kept unlabeled so that it would not produce an NMR signal, but its effect on the spectrum of IL-15α could be observed. A total of 22 residues in the IL-15Rα receptor had significant chemical shift changes in the IL-15Rα/IL-15 cocomplex spectrum compared to the unbound receptor spectrum.

For the present work, full assignment of the complex is not required for our analysis since the goal is to simply map the IL-15 binding site on IL-15Rα qualitatively. If a given residue has the same resonance in the free and complexed IL-15Rα (e.g., Ser61 at 8.6, 113 ppm), that is an indication that its chemical environment does not change upon complex formation, indicating that the residue is not involved in IL15 binding. Conversely, if the chemical shift of a given residue is not identical in the free and complexed protein, this indicates that it is affected by IL-15 binding. In this fashion, it is not required to know exactly which chemical shift it assumes in the complex, but rather that it is different from the free one.

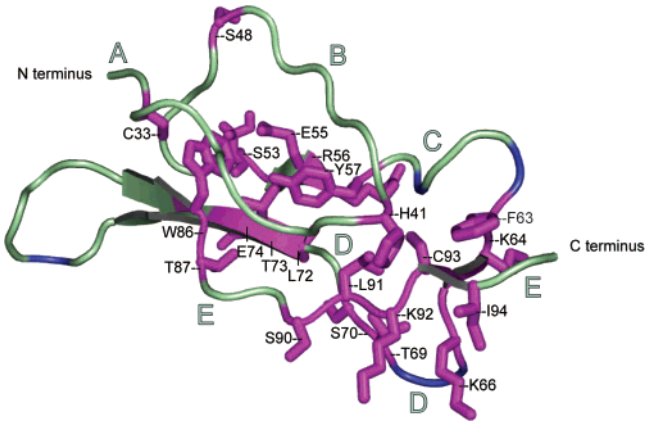

FIGURE 4: Mapping of residues with significant chemical shift differences on the NMR structure of IL-15Rα. Residues with significant chemical shift differences were mapped onto a ribbon representation of the structure of IL-15Rα (magenta), and residues that could not be seen in the NMR spectra are shown (blue). Residues were determined to be significant with chemical shift changes greater than 0.05 ppm in the proton dimension, and 0.2 ppm in the nitrogen dimension.

Location of NMR Chemical Shifts on the IL-15Rα Structure. Residues of IL-15Rα exhibiting significant chemical shift changes between the free receptor spectrum and the cocomplex spectrum were mapped onto the structure of IL-15Rα (Figure 4). Chemical shift differences were mostly localized on the C, D, and E strands of IL-15Rα. Specifically, residues Arg56, Tyr57, Phe63, Lys64, Lys66, Thr69, Ser70, and Leu72 on strands C and D were significant, likely contributing to the interface with IL-15. These strands have been predicted to interact with IL-15 from previous modeling of the receptor/cytokine interface (*27*). Residues Arg65, Ala67, Gly68, and Ser71 on the D strand were among the residues not visible in the NMR spectrum (Figure 4, blue). However these residues are likely involved in IL-15 binding given that they are surrounded by significantly shifted residues on the D strand; however we cannot predict this with absolute certainty. Interestingly, some chemical shift differences were seen from residues on the E strand, which has not previously been predicted to be involved in IL-15, in particular residues Ser90, Lys92, and Ile94.

Several residues were determined to have chemical shifts differences that were not near the predicted IL-15Rα/IL-15 binding interface (Figure 4). Residue His41 on the B strand is located above shifted residues Leu91, Lys92, and Cys93 on the E strand; therefore the chemical environment may be altered due to its proximity to the chemically shifted residues below it, and not necessarily because of a direct interaction. In addition, residues Ser48 and Cys33 are located on the top of the receptor, distal to the predicted interface with IL-15, suggesting that there may be overall conformational changes in the receptor upon ligand binding. These distal residues whose chemical environment is perturbed upon IL-15 binding likely indicate that the IL-15Rα sushi domain has regions of flexibility that are indirectly affected by ligand interaction, either through rigidification or the adoption of alternate conformations. The IL-15Rα contains a significant amount of "loop" structure that is often flexible. Such an observation is entirely consistent with the premise that proteins "breathe" in solution, and highlights the unique ability of NMR to measure structural changes that would not be apparent from a crystal structure.

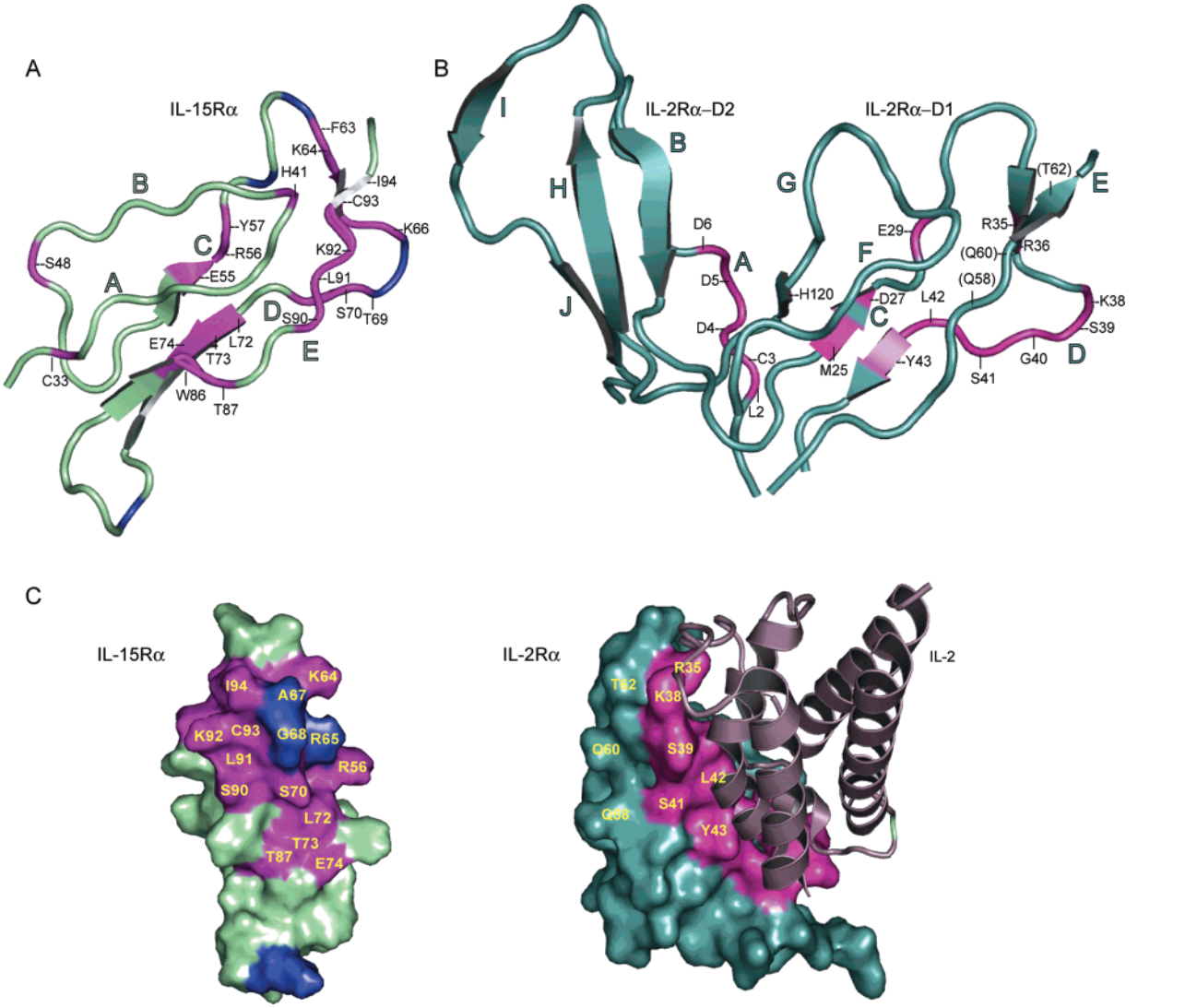

FIGURE 5: Chemical shifts mapped on to IL-15Rα NMR structure and comparison to contacts derived from the crystal structure of IL-2/IL-2Rα. Residues with significant chemical shift differences were mapped onto a ribbon representation of the IL-15Rα NMR structure (A, magenta). IL-2Rα from the IL-2 crystal structure is shown with residues that are in direct contact with IL-2 in magenta (B). Molecular surface representations of IL-15Rα with chemical shift mapping results depicted as magenta surface area (C) and IL-2Rα with contact residues as magenta surface area and IL-2 (purple) bound (D).

A new resonance appears at 10.6/133.5 ppm that likely belongs to a tryptophan side chain (Ne). Intriguingly, the Trp86 side chain peak has low intensity in the free protein, suggesting chemical exchange between different conformations or solvent exchange. Complex formation might stabilize a specific conformation and limit the exchange phenomena, leading to the appearance of a second, more intense peak representing this stabilized form. The Trp86 side chain is part of a hydrophobic patch of residues those chemical shift changes upon complex formation, in accordance with the above hypothesis.

Comparisons of IL-15Rα and IL-2Rα. To address the question of similarities in IL-15Rα and IL-2Rα cytokine recognition, we compared the location of residues on the α receptors that form the interface with IL-15 and IL-2 respectively. First, we generated a structure-based alignment of IL-15Rα onto domain 1 of IL-2Rα to overlay analogous strands. The core rmsd was 2.652 Å using residues 22−47, and 53−64 from IL-15Rα, and residues 104−121 from IL-2Rα. We then compared the NMR chemical shift map of residues on IL-15Rα affected by IL-15 binding (Figure 5A) to residues on IL-2Rα that make contacts with IL-2 in the crystal structure (Figure 5B) (*37*). Significant chemical shift differences on IL-15Rα were located on strands C, D, and to a lesser extent E. The contact residues of IL-2Rα are located primarily on the D, C, and G strands of sushi domain 1, and on the A strand of sushi domain 2. Therefore IL-2Rα does not use the E strand of sushi domain 1 to make contacts with IL-2, so we attribute the chemical shifts on strand E of IL-15Rα to indirect effects propagated from direct interaction with strand C and D.

Model of IL-15Rα/IL-15 Binding Interaction. To further probe the relationship between residues used by the different α receptors for cytokine binding, we modeled where IL-15 would contact IL-15Rα if the binding mode were exactly analogous to that of IL-2 binding to IL-2Rα. We could then compare the resultant IL-15Rα/IL-15 binding interface to the experimentally determined binding footprint as measured by chemical shift mapping. Our approach was to first generate a structural model of IL-15 based on a sequence alignment to IL-2 (see Experimental Procedures). We then individually superimposed the NMR structure of IL-15Rα

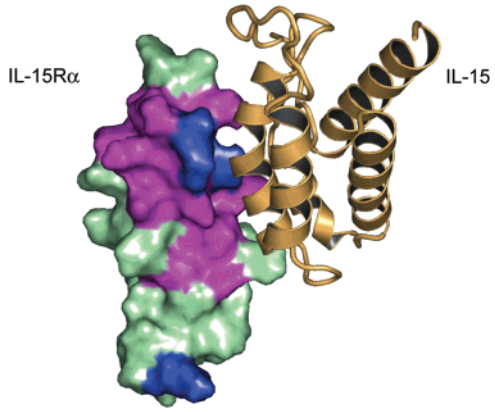

FIGURE 6: Modeling of the IL-15/IL-15Rα interaction based on the IL-2/IL-2Rα docking mode. IL-15 was modeled based on a sequence alignment to IL-2. The model of IL-15 (orange) and structure of IL-15Rα (green) were positioned relative to each other based on their individual superposition onto IL-2 and IL-2Rα respectively, from the IL-2Rα/IL-2 crystal structure.

and the IL-15 homology model onto domain 1 of IL-2Rα and IL-2, respectively, from the IL-2Rα/IL-2 complex crystal structure (Figure 6). The core rmsd achieved for the positioning of IL-15 onto IL-2 was 1.093 Å using residues 1−76, and 95−114 from IL-15, and residues 13−50, 55−73, 81−95, and 113−132 from IL-2. Based on this positioning, it is clear that there is a large degree of overlap between the IL-15 and IL-2 binding footprints on the α receptors. Also, there appear to be charge−charge complementarities between the IL-15 model and the IL-15Rα binding site as observed previously (*27*), although the crudeness of the modeling prevents us from speculating about pairwise contacts. However, if IL-15 were binding to IL-15Rα identical to the "IL-2" mode it would most likely not give rise to the strong chemical shifts we see in the E strand. We conclude that the IL-15/IL-15Rα interaction is highly similar to the IL-2/IL-2Rα interaction, and that the E strand chemical shifts are most likely a result of indirect effects propagated away from the C and D strand interface, or conformational changes upon IL-15 binding.

The interface of chemically shifted residues along strands C and D positions IL-15Rα as a cap on top of IL-15. This mode of docking allows for the trans-presentation of IL-15 by IL-15Rα, where the flexible stalk of IL-15Rα, which would extend from the C terminus of the receptor toward the cell membrane, is away from the predicted IL-15 binding site.

DISCUSSION

The question of how IL-15 interacts with its high affinity receptor IL-15Rα is crucial to understanding the unique biology of this complex, in particular, the ability of IL-15Rα to present IL-15 in trans to cells expressing IL-2Rβ and γ_c. The principal novelties of the results we report here are (1) a robust general method to produce recombinant, biologically active IL-15/IL-15Rα complexes and (2) a study of the IL-15 interaction with IL-15Rα receptor by NMR chemical shift mapping experiments.

With respect to the first novelty, the difficulties in recombinant expression of IL-15 are consistent with previous observations that although *in vivo* IL-15 mRNA transcripts are widely expressed, protein expression is tightly regulated. Our suspicion was that IL-15 protein may be unstable or aggregation prone at high concentrations by itself. The very high affinity of IL-15Rα for IL-15 almost insures that there would be very little free IL-15 in the extracellular millieu, and that most of the IL-15 secreted in a paracrine fashion by T cells exists in complex with its α receptor, possibly in a more biochemically stable form. The instability of free IL-15 complicates reliable binding measurements between free IL-15 and IL-15Rα due to the imprecision in quantitation of biologically active IL-15. We previously noted an almost identical situation with viral IL-6, which was unstable as a free protein, but highly stable and well-behaved in complex with its receptor gp130 (*44*).

In this paper we present a method of IL-15 expression where IL-15 is stabilized by IL-15Rα as it is being secreted from yeast, and therefore is stable in solution. In addition, IL-15 can be produced without tags, as the IL-15Rα hexahistine tag allows for Ni-NTA column purification of the high affinity complex. Our observation that recombinant IL-15 is stabilized when in the presence of its high affinity IL-15Rα receptor is in agreement with how IL-15 is thought to be expressed *in vivo*, bound to either cell surface or soluble shed IL-15Rα receptor and not unbound in cell supernatants (*22*, *45*). Additionally we demonstrate the biological activity of the IL-15Rα/IL-15 complexes in stimulating the proliferation and survival of MP CD8 T cells, and in particular the enhanced potency of these complexes as compared to IL-15 alone. This result supports the idea that biologically relevant IL-15 is likely found in complex with its α receptor.

With regard to the IL-15Rα/IL-15 interaction, we demonstrate that IL-15Rα utilizes two of the same strands (C and D) as IL-2Rα based on chemical shift mapping and a structure-based alignment onto IL-2Rα. Therefore, the primary interaction regions of both IL-15Rα and IL-2Rα are similar. To address this, we modeled the IL-15Rα/IL-15 interaction as if it were exactly analogous to IL-2Rα/IL-2. For this experiment, we superimposed IL-15Rα and a model of IL-15 in the IL-2Rα/IL-2 docking mode. In this binding mode, the residues on the E strand of IL-15Rα that show chemical shift changes do not appear to be in close enough proximity to IL-15 to show chemical shift changes on the receptor. A caveat to this type of analysis is that one cannot accurately predict NMR chemical shift changes from structural models, but one can use both types of information as structural constraints in binding site prediction, as we do here. The chemical shifts of residues along strand E of IL-15Rα, as well as sites in the receptor not near the predicted IL-15 binding interface, suggest overall structural adjustments of flexible regions, occurring in the receptor upon ligand binding. Ultimately, the nature of the chemical shifts on IL-15Rα that are not at the predicted interface will best be understood by a complete structure of the IL-15Rα/IL-15 complex and the quaternary structure of IL-15/IL-15Rα/IL-2Rβ/γ_c.

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REFERENCES

1. Burton, J. D., Bamford, R. N., Peters, C., Grant, A. J., Kurys, G., Goldman, C. K., Brennan, J., Roessler, E., and Waldmann, T. A. (1994) A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells, *Proc. Natl. Acad. Sci. U.S.A. 91*, 4935−4939.
2. Bamford, R. N., Grant, A. J., Burton, J. D., Peters, C., Kurys, G., Goldman, C. K., Brennan, J., Roessler, E., and Waldmann, T. A. (1994) The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells, *Proc. Natl. Acad. Sci. U.S.A. 91*, 4940−4944.
3. Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M. A., Ahdieh, M., et al. (1994) Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor, *Science 264*, 965−968.
4. Carson, W. E., Fehniger, T. A., Haldar, S., Eckhert, K., Lindemann, M. J., Lai, C. F., Croce, C. M., Baumann, H., and Caligiuri, M. A. (1997) A potential role for interleukin-15 in the regulation of human natural killer cell survival, *J. Clin. Invest. 99*, 937−943.
5. Cooper, M. A., Bush, J. E., Fehniger, T. A., VanDeusen, J. B., Waite, R. E., Liu, Y., Aguila, H. L., and Caligiuri, M. A. (2002) In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells, *Blood 100*, 3633−3638.
6. Becknell, B., and Caligiuri, M. A. (2005) Interleukin-2, interleukin-15, and their roles in human natural killer cells, *Adv Immunol 86*, 209−239.
7. Zhang, X., Sun, S., Hwang, I., Tough, D. F., and Sprent, J. (1998) Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15, *Immunity 8*, 591−599.
8. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J., and Marrack, P. (2000) Control of homeostasis of CD8+ memory T cells by opposing cytokines, *Science 288*, 675−678.
9. Marrack, P., Bender, J., Hildeman, D., Jordan, M., Mitchell, T., Murakami, M., Sakamoto, A., Schaefer, B. C., Swanson, B., and Kappler, J. (2000) Homeostasis of alpha beta TCR+ T cells, *Nat. Immunol. 1*, 107−111.
10. Weng, N. P., Liu, K., Catalfamo, M., Li, Y., and Henkart, P. A. (2002) IL-15 is a growth factor and an activator of CD8 memory T cells, *Ann. N.Y. Acad. Sci. 975*, 46−56.
11. Becker, T. C., Wherry, E. J., Boone, D., Murali-Krishna, K., Antia, R., Ma, A., and Ahmed, R. (2002) Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells, *J. Exp. Med. 195*, 1541−1548.
12. Ohteki, T. (2002) Critical role for IL-15 in innate immunity, *Curr. Mol. Med. 2*, 371−380.
13. Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994) Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15, *EMBO J. 13*, 2822−2830.
14. Anderson, D. M., Kumaki, S., Ahdieh, M., Bertles, J., Tometsko, M., Loomis, A., Giri, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., et al. (1995) Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes, *J. Biol. Chem. 270*, 29862−29869.
15. Giri, J. G., Kumaki, S., Ahdieh, M., Friend, D. J., Loomis, A., Shanebeck, K., DuBose, R., Cosman, D., Park, L. S., and Anderson, D. M. (1995) Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor, *EMBO J. 14*, 3654−3663.
16. Budagian, V., Bulanova, E., Paus, R., and Bulfone-Paus, S. (2006) IL-15/IL-15 receptor biology: A guided tour through an expanding universe, *Cytokine Growth Factor Rev. 17*, 259−280.
17. Norman, D. G., Barlow, P. N., Baron, M., Day, A. J., Sim, R. B., and Campbell, I. D. (1991) Three-dimensional structure of a complement control protein module in solution, *J. Mol. Biol. 219*, 717−725.
18. Wang, X., Rickert, M., and Garcia, K. C. (2005) Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gammac receptors, *Science 310*, 1159−1163.
19. Wei, X., Orchardson, M., Gracie, J. A., Leung, B. P., Gao, B., Guan, H., Niedbala, W., Paterson, G. K., McInnes, I. B., and Liew, F. Y. (2001) The Sushi domain of soluble IL-15 receptor alpha is essential for binding IL-15 and inhibiting inflammatory and allogenic responses in vitro and in vivo, *J. Immunol. 167*, 277−282.
20. Mortier, E., Quemener, A., Vusio, P., Lorenzen, I., Boublik, Y., Grotzinger, J., Plet, A., and Jacques, Y. (2006) Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins, *J. Biol. Chem. 281*, 1612−1619.
21. Rickert, M., Boulanger, M. J., Goriatcheva, N., and Garcia, K. C. (2004) Compensatory energetic mechanisms mediating the assembly of signaling complexes between interleukin-2 and its alpha, beta, and gamma(c) receptors, *J. Mol. Biol. 339*, 1115−1128.
22. Dubois, S., Mariner, J., Waldmann, T. A., and Tagaya, Y. (2002) IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells, *Immunity 17*, 537−547.
23. Schluns, K. S., Klonowski, K. D., and Lefrancois, L. (2004) Transregulation of memory CD8 T-cell proliferation by IL-15Ralpha+ bone marrow-derived cells, *Blood 103*, 988−994.
24. Burkett, P. R., Koka, R., Chien, M., Chai, S., Boone, D. L., and Ma, A. (2004) Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis, *J. Exp. Med. 200*, 825−834.
25. Sandau, M. M., Schluns, K. S., Lefrancois, L., and Jameson, S. C. (2004) Cutting edge: transpresentation of IL-15 by bone marrow-derived cells necessitates expression of IL-15 and IL-15R alpha by the same cells, *J. Immunol. 173*, 6537−6541.
26. Johnston, J. A., Bacon, C. M., Finbloom, D. S., Rees, R. C., Kaplan, D., Shibuya, K., Ortaldo, J. R., Gupta, S., Chen, Y. Q., Giri, J. D., et al. (1995) Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15, *Proc. Natl. Acad. Sci. U.S.A. 92*, 8705−8709.
27. Lorenzen, I., Dingley, A. J., Jacques, Y., and Grotzinger, J. (2006) The structure of the interleukin-15 alpha receptor and its implications for ligand binding, *J. Biol. Chem. 281*, 6642−6647.
28. Maynard, J., Petersson, K., Wilson, D. H., Adams, E. J., Blondelle, S. E., Boulanger, M. J., Wilson, D. B., and Garcia, K. C. (2005) Structure of an autoimmune T cell receptor complexed with class II peptide-MHC: insights into MHC bias and antigen specificity, *Immunity 22*, 81−92.
29. Shusta, E. V., Kieke, M. C., Parke, E., Kranz, D. M., and Wittrup, K. D. (1999) Yeast polypeptide fusion surface display levels predict thermal stability and soluble secretion efficiency, *J. Mol. Biol. 292*, 949−956.
30. Robinson, A. S., Hines, V., and Wittrup, K. D. (1994) Protein disulfide isomerase overexpression increases secretion of foreign proteins in Saccharomyces cerevisiae, *Bio/Technology 12*, 381−384.
31. Kieper, W. C., Tan, J. T., Bondi-Boyd, B., Gapin, L., Sprent, J., Ceredig, R., and Surh, C. D. (2002) Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells, *J. Exp. Med. 195*, 1533−1539.
32. Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J., and Surh, C. D. (2002) Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells, *J. Exp. Med. 195*, 1523−1532.
33. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes, *J. Biomol. NMR 6*, 277−293.
34. Goddard, T. D., and Kneller, D. G. (1999) *SPARKY 3*, University of California, San Francisco.
35. Bernard, J., Harb, C., Mortier, E., Quemener, A., Meloen, R. H., Vermot-Desroches, C., Wijdeness, J., van Dijken, P., Grotzinger, J., Slootstra, J. W., Plet, A., and Jacques, Y. (2004) Identification of an interleukin-15alpha receptor-binding site on human interleukin-15, *J. Biol. Chem. 279*, 24313−24322.
36. Sali, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints, *J. Mol. Biol. 234*, 779−815.
37. Rickert, M., Wang, X., Boulanger, M. J., Goriatcheva, N., and Garcia, K. C. (2005) The structure of interleukin-2 complexed with its alpha receptor, *Science 308*, 1477−1480.
38. Krissinel, E., and Henrick, K. (2004) Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 60*, 2256−2268.

39. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 60*, 2126−2132.
40. DeLano, W. L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA. http://www.pymol.org.
41. Pettit, D. K., Bonnert, T. P., Eisenman, J., Srinivasan, S., Paxton, R., Beers, C., Lynch, D., Miller, B., Yost, J., Grabstein, K. H., and Gombotz, W. R. (1997) Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modeling, *J. Biol. Chem. 272*, 2312−2318.
42. Rubinstein, M. P., Kovar, M., Purton, J. F., Cho, J. H., Boyman, O., Surh, C. D., and Sprent, J. (2006) Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}, *Proc. Natl. Acad. Sci. U.S.A. 103*, 9166−9171.
43. Stoklasek, T. A., Schluns, K. S., and Lefrancois, L. (2006) Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo, *J. Immunol. 177*, 6072−6080.
44. Chow, D., He, X., Snow, A. L., Rose-John, S., and Garcia, K. C. (2001) Structure of an extracellular gp130 cytokine receptor signaling complex, *Science 291*, 2150−2125.
45. Bamford, R. N., Battiata, A. P., Burton, J. D., Sharma, H., and Waldmann, T. A. (1996) Interleukin (IL) 15/IL-T production by the adult T-cell leukemia cell line HuT-102 is associated with a human T-cell lymphotrophic virus type I region /IL-15 fusion message that lacks many upstream AUGs that normally attenuates IL-15 mRNA translation. *Proc. Natl. Acad. Sci. U.S.A. 93*, 2897−2902.

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