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Dual Mechanism of Interleukin-3 Receptor Blockade by an Anti-Cancer Antibody

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SUMMARY

Interleukin-3 (IL-3) is an activated T cell product that bridges innate and adaptive immunity and contributes to several immunopathologies. Here, we report the crystal structure of the IL-3 receptor α chain (IL3Rα) in complex with the anti-leukemia antibody CSL362 that reveals the N-terminal domain (NTD), a domain also present in the granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and IL-13 receptors, adopting unique "open" and classical "closed" conformations. Although extensive mutational analyses of the NTD epitope of CSL362 show minor overlap with the IL-3 binding site, CSL362 only inhibits IL-3 binding to the closed conformation, indicating alternative mechanisms for blocking IL-3 signaling. Significantly, whereas "open-like" IL3Rα mutants can simultaneously bind IL-3 and CSL362, CSL362 still prevents the assembly of a higher-order IL-3 receptor-signaling complex. The discovery of open forms of cytokine receptors provides the framework for development of potent antibodies that can achieve a "double hit" cytokine receptor blockade.

INTRODUCTION

Interleukin 3 (IL-3) is a cytokine produced largely by antigen-activated T cells that links immunity to the hemopoietic system and plays a significant role in leukemia as well as several immunopathologies (Hercus et al., 2013). Through actions on several cell types, IL-3 contributes to allergic inflammation, autoimmune diseases, and oncogenesis (Korpelainen et al., 1993; Broughton et al., 2012). Importantly, leukemic stem cells from patients with acute myeloid leukemia (AML) (Jordan et al., 2000) and chronic myeloid leukemia (CML) (Nievergall et al., 2014) overexpress the IL-3 receptor α chain (IL3Rα), and this is associated with a poor prognosis in AML (Testa et al., 2002). Understanding how the IL-3 receptor signals and designing strategies to optimally target the IL-3 receptor has become a pressing need.

The IL-3 receptor belongs to the βc family of heterodimeric receptors that includes the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 receptors and is part of the type I cytokine receptor superfamily. The βc family utilizes specific subunits (α) that bind the cognate cytokine as well as the shared subunit (βc) that is essential for signaling (Hercus et al., 1994). In the GM-CSF receptor, a dodecamer assembly brings βc-associated JAK2 molecules on hexamer complexes into close proximity to initiate receptor signaling (Hansen et al., 2008) with a similar mechanism proposed for IL-3 and IL-5 receptor signaling (Patino et al., 2011; Kusano et al., 2012; Broughton et al., 2012).

The IL3Rα N-terminal domain (NTD) allows optimal IL-3 binding but is not essential for IL-3 signaling (Barry et al., 1997; Chen et al., 2009). Paradoxically, monoclonal antibody (mAb) 7G3 recognizes the IL3Rα NTD (Sun et al., 1996; Barry et al., 1997) and completely blocks IL-3 binding and signaling. We recently showed that mAb 7G3 eliminates engrafted human AML stem cells in a mouse model of human AML (Jin et al., 2009). mAb 7G3, now humanized and designated as CSL362 (Busfield et al., 2014), is entering clinical trials for the treatment of patients with AML (ClinicalTrials.gov identifier: NCT01632852). We have now used a Fab fragment of CSL362 to solve the structure of IL3Rα and reveal the underlying mechanisms of CSL362-mediated IL-3 receptor blockade. First, we observed IL3Rα NTD in two distinct conformations, "open" and "closed," suggesting that the NTD is mobile and may modulate IL-3 signaling. Second, CSL362 and IL-3 utilize functionally distinct binding sites on IL3Rα, and using "open-like" IL3Rα mutants, we showed that CSL362 can inhibit IL-3 signaling without inhibiting IL-3 binding. Third, when combined with direct competition for IL-3 binding to the closed IL3Rα, it is clear that CSL362 has dual mechanisms leading to IL-3 receptor blockade. Finally, we generated a model

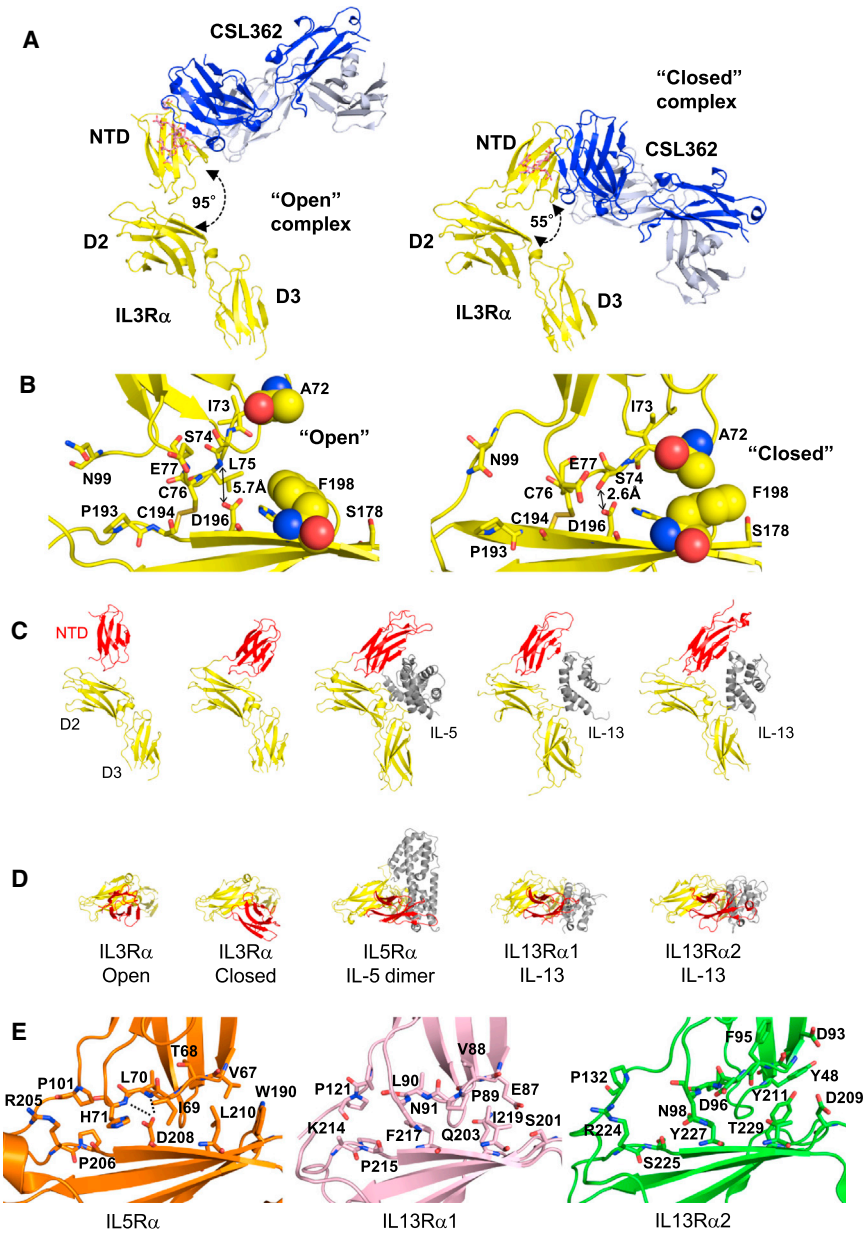


Figure 1. Structure of the IL3Rα:CSL362 Fab Complex

(A) Crystal structure of the IL3Rα:CSL362 binary complex reveals two conformational states: open and closed. IL3Rα in yellow, CSL362 heavy chain in dark blue, CSL362 light chain in light blue, and glycan molecules represented as pink sticks.

(B) View of the interface between IL3Rα NTD and D2 in the open and closed complexes. Key features are indicated, including the C76-C194 disulfide bond and the distance between S74 and D196.

(C and D) "Side on" (C) and "top down" (D; when looking down onto the membrane surface) views of related cytokine receptors, IL5Rα (PDB ID: 3QT2; Patino et al., 2011), IL13Rα1 (PDB ID: 3BPO; LaPorte et al., 2008), and IL13Rα2 (PDB ID: 3LB6; Lupardus et al., 2010). Receptors are aligned via their D2-D3 domains to the closed form of IL3Rα and colored yellow with NTD domains red and cytokines gray.

(E) Comparison of the NTD-D2 interface for IL5Rα (orange), IL13Rα1 (pink), and IL13Rα2 (green); same alignment as in (C) and (D) and same view as for IL3Rα in (B). In IL5Rα, D208 hydrogen bonds to the amide nitrogen of both L70 and H71.

See also Figures S1–S4 and Table S1.

of the functional IL-3 receptor based on these structural, functional, and homology studies, which support the requirement of a higher-order assembly for IL-3 receptor signaling. These results have important implications for understanding cytokine receptor signaling and for guiding the development of novel and potent anti-cytokine-receptor antibodies.

RESULTS

The Structure of the IL3Rα:CSL362 Complex Reveals Open and Closed Conformations of IL3Rα

Soluble IL3Rα (sIL3Rα) was bound to the Fab fragment of CSL362 and the crystal structure of the binary complex determined (Figure S1; Table S1). For complete crystallization details, see Broughton et al. (2014). Two independent forms of the binary complex were observed in the asymmetric unit of the unit cell, an open and a closed form, each of which comprise a sIL3Rα molecule and a CSL362 Fab (Figure 1A). In both forms, CSL362 Fab binds to the NTD of IL3Rα, consistent with cell binding studies (Figure S2).

The IL3Rα structure reveals a well-defined immunoglobulin-like NTD that adopts a typical fibronectin type III (FnIII) fold followed by two more FnIII domains (D2 and D3) that form the cytokine receptor module (CRM) that is typical for this class of receptors (Broughton et al., 2012). The major difference between the two complexes is the angle between the NTD and D2, which differs by ~40° (Figures 1A and S3A; see the Supplemental Results). The position of the NTD is constrained by the disulfide bond between C76 (NTD) and C194 (D2), a hydrogen bond between S74 (NTD) and D196 (D2), and van der Waals (vdW) interactions between A72 (NTD) and F198 (D2; Figure 1B). Although it is possible that the two IL3Rα conformations are a result of crystal packing, the biological data presented below suggest a functional purpose for the flexibility of the NTD-D2 linker region that results in the receptor adopting both conformations and possibly a dynamic continuum between them.

Unique and Conserved Structural Features of IL3Rα

The ligand-free IL3Rα structures were compared to structures for other βc receptors, including two structures of IL5Rα in

complex with IL-5 (Protein Data Bank [PDB] IDs: 3QT2 and 3VA2; Patino et al., 2011; Kusano et al., 2012), and our partial structure of GMRα in the GM-CSF receptor ternary complex (PDB ID: 3CXE; Hansen et al., 2008). Whereas GMRα structural data are largely restricted to D3, the closed conformation of IL3Rα clearly resembles the "wrench-like" fold observed in IL5Rα bound to IL-5 (Figure 1C). The two IL5Rα binary complexes observed in the asymmetric unit of 3QT2 and the one in 3VA2 are very similar (Cα root-mean-square-deviation [rmsd] = ~0.5 Å); hence all comparisons with IL3Rα have been carried out using 3QT2. The CRMs of IL3Rα and IL5Rα superimpose closely with the biggest difference being a lengthy loop region in IL5Rα (E120–Y129) that is proximal to the D2-D3 linker, protruding outward toward the cytokine-binding interface. The core β sheet structure of the NTDs is largely maintained between IL3Rα and IL5Rα (Cα rmsd = 1.6 Å); however, in IL5Rα, the four-stranded β sheet at the distal tip from the CRM twists toward the cytokine and the loops in this region are considerably longer (Figure S4A).

A 3D structural comparison of IL3Rα bound to CSL362 using the PDB eFold server (http://www.ebi.ac.uk/msd-srv/ssm) revealed similarity with other type I cytokine receptors. Outside the βc family, the closed conformation of IL3Rα has the strongest similarity with the IL-13 receptors, IL13Rα1 and IL13Rα2, that bind IL-13 in a pincer-like grip between the NTD and the CRM (Figures 1C and 1D; PDB IDs: 3BPO [LaPorte et al., 2008] and 3LB6 [Lupardus et al., 2010]). The core β sheet structure of the IL3Rα NTD overlays well with the NTDs of IL13Rα1 and IL13Rα2 (Cα rmsd = 1.6 Å for both); however, loop regions at the CRM distal tip of the NTD in IL13Rα1/IL13Rα2 are lengthy compared to the relatively compact loops of IL3Rα. Although the NTD-D2 and D2-D3 elbow angles are similar between the closed IL3Rα conformation, IL5Rα, IL13Rα1, and IL13Rα2, the IL3Rα NTD is twisted away from the D2-D3 axis by ~30° compared to the other receptors so that it is not sitting directly above D2 (Figure 1D). See the Supplemental Results for additional comparisons.

Residues located at the NTD-D2 interface for IL3Rα (Figure 1B) and IL5Rα (Figure 1E) show some conservation: e.g., IL5Rα D208 (analogous to IL3Rα D196) hydrogen bonds to L70 and H71 (NTD) whereas V67 (NTD) makes vdW interactions with W190 and L210 (D2). There is little sequence identity between IL3Rα and IL13Rα1/IL13Rα2 in the NTD-D2 interface; however, a number of hydrogen bonds and vdW interactions may serve a similar role in orientating the NTD (Figures 1B and 1E).

CSL362 Binds to a 15 Amino Acid Epitope in the IL3Rα NTD

The CDR loops from CSL362 interact with strand D and portions of the BC, DE, and FG loops of IL3Rα NTD (Figures 2A, S1A, and S1B; see the Supplemental Results) and the CDR interface with IL3Rα in both open and closed conformations are essentially identical (Cα rmsd = 0.5 Å; Figure 2B). The interacting residues are shown in Figures 2C and 2D. Although closely associated with the CSL362 epitope, the N-linked glycosylation at N80 of IL3Rα is not required for CSL362 binding (see the Supplemental Results; Figures S3C–S3E).

All CDR loops of CSL362 interact with the IL3Rα NTD, except for the light-chain CDR2 loop, and are detailed in Table S2. The heavy (H) and light (L) chains of CSL362 form ten hydrogen bonds and three salt bridges with IL3Rα (Figure 2D; Table S2). The side-chain hydroxyl of CDR1H Tyr33H forms hydrogen bonds between the amide nitrogen atom of V85 and the carbonyl of F90. CDR2H Asp50H forms a salt bridge with the guanidine moiety of R84, and two salt bridges are formed between CDR3H Arg103 and both D49 and E51. CDR3L residues Tyr98L, Ser99L, Tyr100L, and Tyr102L hydrogen bond to S59 through both main-chain and side-chain interactions whereas two hydrogen bonds are formed between the side chains of Tyr102L and E51 and R84. CDR3L residues Tyr98L, Ser99L, and Tyr100L pack against the main chain of residues P61, M60, S59, Y58, D57, and A56. These interactions define the structural epitope of CSL362 binding to IL3Rα and are consistent with the CSL362-interacting footprint determined in solution using hydrogen/deuterium exchange (see the Supplemental Results).

Functional Characterization of the CSL362 Epitope on the IL3Rα NTD

To define the productive interactions in the CSL362 epitope, we assessed the binding of mAb 7G3 (from which CSL362 was derived) to a panel of IL3Rα mutants by flow cytometry. This analysis identified E51 and S59 as key determinants of 7G3 binding as well as revealing a role for an adjacent residue, P88 (Figure 2E). The interactions were quantified by saturation-binding analysis of radioiodinated CSL362 to IL3Rα mutants. Mutation of E51 and R84 abolished CSL362 binding whereas mutation of Y58, S59, P61, and P88 reduced CSL362 binding (Table S3). Surface plasmon resonance (SPR) analysis of CSL362 binding to sIL3Rα mutants also identified E51 as a critical determinant whereas mutation of D49, Y58, S59, and P61 reduced CSL362 binding (Table S3).

Identification and Characterization of the IL-3 Binding Site in IL3Rα

To explore the mechanism of action for CSL362, we investigated the location of the IL-3 binding site in IL3Rα. We constructed models of the IL-3:IL3Rα binary complex based on (1) the open and closed conformations of IL3Rα (Figure 1A), (2) published mutagenesis data (Broughton et al., 2012), and (3) automated cytokine-receptor docking (model details in Supplemental Results). In the open model, IL-3 can adopt multiple orientations, whereas in the closed model, IL3Rα wraps around the cytokine (Figure 3A), reminiscent of the IL5Rα and IL13Rα1/IL13Rα2 interactions with cytokine (Figures 1C, 1D, and S4A). Superimposing the bound CSL362 Fab on these models indicates that CSL362 and IL-3 are potentially able to bind simultaneously to IL3Rα in the open model, whereas in the closed model, CSL362 overlaps the docked IL-3 (Figure 3B). The closed IL-3:IL3Rα binary model is very similar to the IL-5:IL5Rα binary complex (Figure S4A) with the four α helices of IL-3 overlapping with helices A, B, C, and D' in IL-5 (Patino et al., 2011; Kusano et al., 2012).

The binding of cytokine to its α subunit was first defined for the two domain growth hormone receptors as site 1 (de Vos et al., 1992). Classical site 1 interactions are predicted to occur between helices A, A', and D of IL-3 and the "elbow" region between D2 and D3 of IL3Rα. This is site 1a in our model (Figures

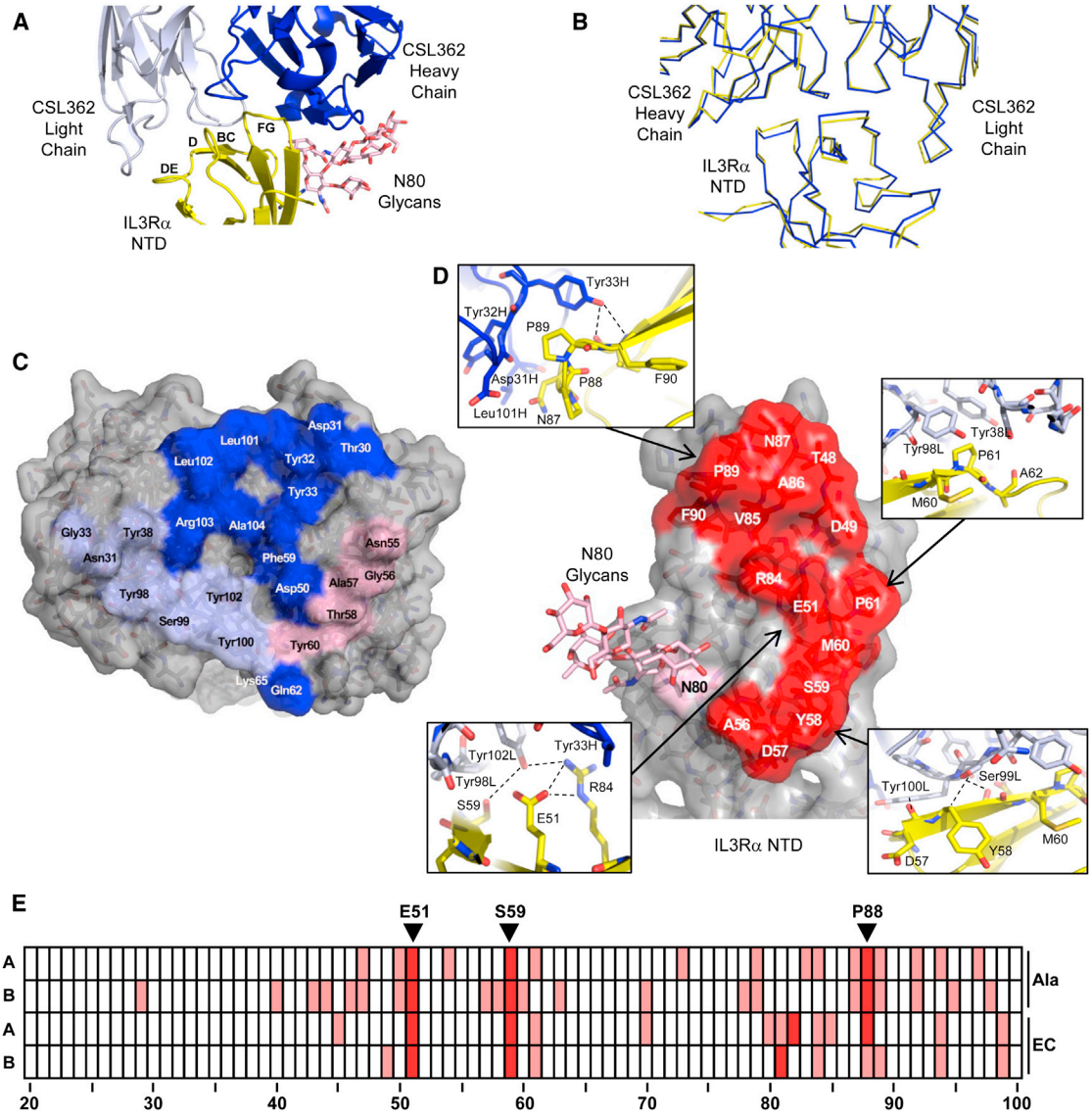


Figure 2. The CSL362 Epitope on IL3Rα

(A) Cartoon representation of the interaction interface between IL3Rα and CSL362, colored as in Figure 1. CSL362-interacting loops and β strands are labeled.

(B) Overlay of the Cα trace of the IL3Rα NTD:CSL362 interface region from the open (yellow) and closed (blue) complexes.

(C) Surface representation of CSL362 showing the IL3Rα footprint on the heavy chain (dark blue) and the light chain (light blue) as well as the IL3Rα N80 glycan contact (pink).

(D) Semitransparent surface view of IL3Rα with residues that interact with CSL362 colored red and the N80-linked glycan in pink. Detailed residue interactions between CSL362 and IL3Rα are highlighted.

(E) Mapping of the CSL362 functional epitope by alanine (Ala) or evolutionary conserved (EC) mutation of IL3Rα residues 20–100 and measuring 7G3 binding by flow cytometry.

Relative 7G3 binding, normalized for protein expression using 9F5 (A) or 107D2 (B) binding, is reported as 75%–100% (open squares), 50%–74% (pink squares), 25%–49% (red squares), and 0%–24% (maroon squares). Data are shown from a representative experiment (n = 3). See also Figures S2–S4 and Tables S2 and S3.

3A, 3C, and 3D) whereas nonclassical contacts between IL-3 and the NTD of IL3Rα are distinguished as site 1b (Figures 3A, 3C, and 3D; details are provided in the Supplemental Results; Table S4). IL-5 and IL-13 interact with their respective α subunits in a similar manner (Figures 1C, 1D, and S4A) with site 1a interactions located in similar regions, but not conserved. For site 1b, vdW interactions dominate for IL3Rα whereas hydrogen bonds and polar interactions dominate for IL5Rα. The IL13Rα1/IL13Rα2 site 1b interactions are comprised of hydrogen bonds between cytokine and receptor, similar to IL5Rα, and vdW interactions between cytokine and receptor, similar to IL3Rα.

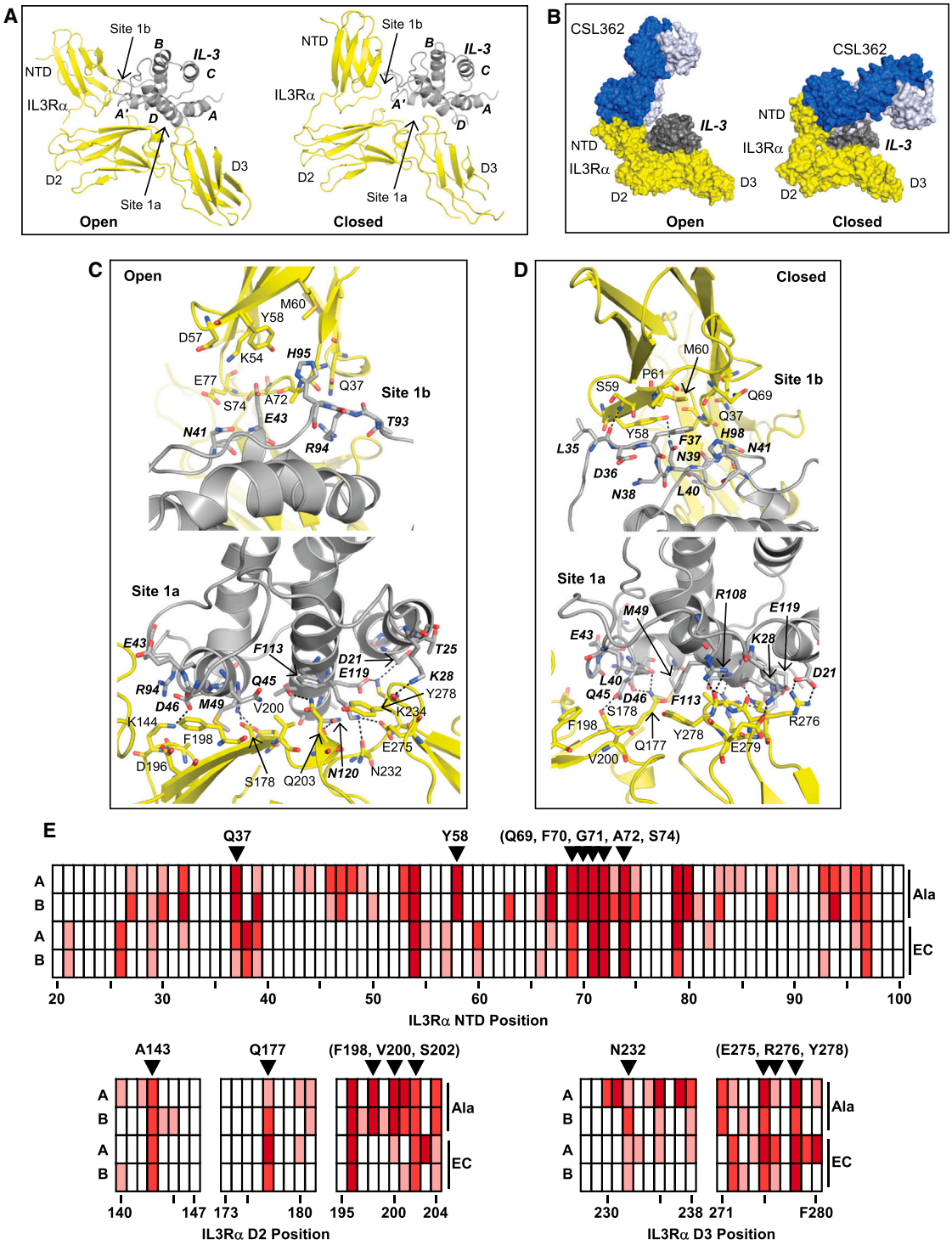


Figure 3. Molecular Modeling of IL-3 Bound to IL3Rα

(A) Docking of IL-3 to the open and closed conformations of IL3Rα colored as in Figure 1. The location of the NTD, D2, D3, and site 1 are indicated, and the IL-3 α helices are labeled.

(B) Overlay of the open and closed IL-3 binary complex models on the respective open and closed IL3Rα:CSL362 crystal complexes with proteins depicted as molecular surface.

(C and D) Details of the IL-3:IL3Rα interface for the open (C) and the closed (D) IL-3 binary complex models, showing site 1a (lower) and site 1b (upper) interactions. Residues in IL-3 are highlighted in bold italics with putative hydrogen bonds or salt bridges displayed as black dashed lines.

(legend continued on next page)

The two major isoforms of IL3Rα are full-length (SP1) and a truncated isoform called SP2 that lacks the NTD (Chen et al., 2009) that by itself has no measurable IL-3 binding (Table S5). We measured the binding of IL-3 to IL3Rα mutants by flow cytometry to assess IL3Rα site 1 interactions and identified residues that contribute to IL-3 binding, including Q37, Y58, Q69, F70, G71, A72, S74, A143, Q177, F198, V200, S202, N232, E275, R276, and Y278 (Figure 3E; see the Supplemental Results). SPR analysis revealed that mutation of Y58, G71, E275, and Y278 abolished measurable IL-3 binding (K_D > 5,000 nM) whereas mutation of Q37, Q69, Q177, F198, V200, S202, and N232 reduced IL-3 binding as did mutation of an adjacent residue A143 (Table S5), confirming a role for these residues in IL-3 binding. All IL3Rα site 1 mutants were able to bind radioiodinated IL-3 with wild-type (WT) affinity when coexpressed on cells with βc (Table S5) consistent with previous studies where reduced ligand binding by the α receptor can be compensated by the presence of βc (Mirza et al., 2010). Despite the overlap of the CSL362 and IL-3 contact surfaces in the IL3Rα NTD, only residue Y58 contributes to both interactions (Tables S3 and S5; Figure S4B). The interactions are otherwise qualitatively and functionally distinct (see the Supplemental Results; Figures S4C–S4F).

CSL362 Blocks Signaling by Preventing the Assembly of a Higher-Order IL-3 Receptor Complex

The open conformation of IL3Rα may allow simultaneous binding of CSL362 and IL-3 (Figure 3B) and suggested that CSL362 may block receptor function through a mechanism independent of cytokine binding. We used our structure of the GM-CSF ternary complex and cytokine-receptor docking to construct IL-3 ternary complex models (see the Supplemental Results; Figures S5A–S5D). Modeling of the open IL-3 receptor hexamer complex suggested that CSL362 binding will not compete for IL-3 binding in *cis* but will clash with IL-3 and IL3Rα in *trans* through the hexamer partner (Figures S5E–S5H), preventing dodecamer complex formation and IL-3 signaling. To test this prediction, we engineered open-like IL3Rα mutants by substitution of IL3Rα D196 with residues that fill the pocket between the NTD and D2 (Figure 4A). These mutants are expected to function with properties similar to the IL3Rα SP2 isoform, which lacks the NTD (Chen et al., 2009). When coexpressed with βc on COS cells, we observed comparable and moderate IL-3 binding affinities for D196L, D196K, or D196R (D196L/D196K/D196R) IL3Rα open-like variants and IL3Rα SP2 (Figure 4B). The binding of CSL362 to the IL3Rα D196 mutants was unchanged (Figure 4B). CSL362 effectively blocked IL-3 binding to COS cells coexpressing βc and WT IL3Rα but only partially blocked IL-3 binding to cells expressing the IL3Rα D196L/D196K/D196R variants and was unable to block IL-3 binding to cells expressing IL3Rα SP2 (Figure 4C). In binding experiments using Eu-labeled IL-3 and Tb-labeled CSL362, only COS cells expressing the IL3Rα D196L/D196K/D196R variants were able to bind both ligands simultaneously (Figure 4D). The results are consistent with the IL3Rα D196L/D196K/D196R variants adopting an open-like conformation that allows concomitant binding of CSL362 and IL-3 (Figure 3B).

Ba/F3 cells coexpressing βc and the IL3Rα variants proliferated in response to IL-3 stimulation with potencies consistent with their IL-3 binding affinities (Figure 4E). Preincubation with CSL362 completely inhibited IL-3-mediated proliferation of Ba/F3 cells expressing all forms of full-length IL3Rα (Figure 4F). CSL362 also blocked IL-3-stimulated STAT5 phosphorylation in Ba/F3 cells expressing WT, D196A, or D196K IL3Rα, but not in cells expressing IL3Rα SP2 (Figure 4G). The observation that CSL362 fully blocks IL-3 signaling despite only partially competing for IL-3 binding to IL3Rα open-like mutants supports a mechanism of action where CSL362 is able to prevent the formation of a higher-order IL-3 receptor complex and is consistent with IL-3 utilizing a dodecameric signaling complex (Figure 5).

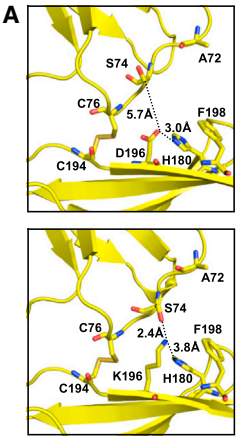
DISCUSSION

We report here the structure of the human IL-3 receptor in complex with a Fab fragment of the neutralizing mAb CSL362, which reveals features of signaling in the type I cytokine receptor superfamily as well as a dual mechanism of antibody-mediated cytokine receptor blockade (Figure 5). The structure of the IL-3 receptor showed two alternative conformations, an open and a closed form based on the orientation of the NTD (Figure 1). This was unexpected as the open conformation has not been seen in the related IL-5 (Patino et al., 2011; Kusano et al., 2012) or IL-13 receptors (LaPorte et al., 2008; Lupardus et al., 2010). Most type I cytokine receptors include a single CRM consisting of two domains with a conserved topology that forms the main surface used to recognize ligand. The α subunits of the βc family, IL13Rα1 and IL13Rα2, represent a group of type I receptors that include an additional NTD, but the exact function of this domain has not been clearly understood.

In the closed conformation of IL3Rα, the orientation and overall topology of the NTD is very similar to the NTDs of IL5Rα and IL13Rα1/IL13Rα2 bound to ligand (Figures 1B and 1C). Our results suggest that the IL3Rα NTD is highly flexible and potentially allows subtle modulation of ligand binding, receptor interactions, and signaling. It is likely that the open and closed conformations represent the dynamic extremes of NTD movement and may also occur in the structurally related GM-CSF, IL-5, and IL-13 α subunits.

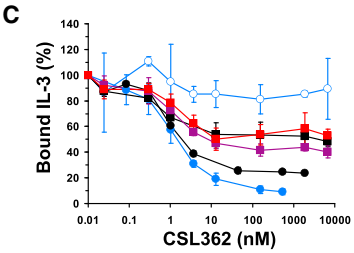
Elucidation of the IL3Rα structure allowed us to investigate how mAb CSL362 blocks IL-3 signaling and the role of the NTD in this process. Epitope-mapping experiments identified E51, S59, and R84 and to a lesser extent D49, Y58, P61, and P88 in the NTD as important residues for CSL362 binding (Figures 2E and S4C–S4F; Table S3). To examine if the CSL362 epitope overlapped with the IL-3 binding site, we constructed

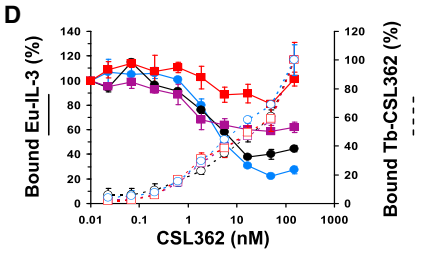
(E) Mapping of the IL-3-binding site by Ala or EC mutation of IL3Rα site 1 residues and measuring IL-3 binding by flow cytometry.
Relative IL-3 binding, normalized for protein expression using 9F5 (A) or 107D2 (B) binding, is reported as 75%–100% (open squares), 50%–74% (pink squares), 25%–49% (red squares), and 0%–24% (maroon squares). Data are shown from a representative experiment (n = 3). See also Figures S3–S5 and Table S4 and S5.

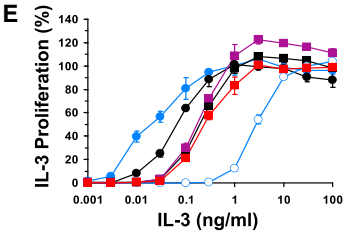


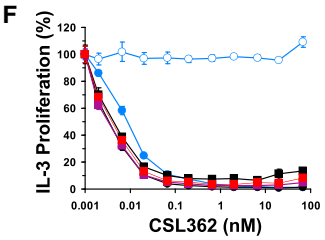
B

IL3Rα	n	IL-3 α+β K_D (nM)	n	CSL362 K_D (nM)	n	9F5 K_D (nM)
Wild Type	15	0.4 ±0.1	6	4.0 ±1.1	6	1.3 ±0.3
SP2	8	5.3 ±3.3		N.B.		N.B.
D196E	4	1.2 ±0.5	2	3.0 ±0.6	3	1.6 ±0.4
D196A	4	1.6 ±0.8	2	2.6 ±0.6	3	1.4 ±0.1
D196S	4	1.0 ±0.4	2	2.7 ±0.8	3	1.5 ±0.5
D196N	4	1.4 ±0.7	2	2.2 ±0.6	3	1.5 ±0.4
D196L	6	4.4 ±1.5	2	2.0 ±0.5	2	1.2 ±0.0
D196Y	6	1.1 ±0.2	2	2.6 ±0.1	2	1.3 ±0.2
D196K	9	4.2 ±2.4	2	2.3 ±0.6	2	1.3 ±0.0
D196R	6	4.4 ±1.5	2	2.4 ±0.2	2	1.3 ±0.1









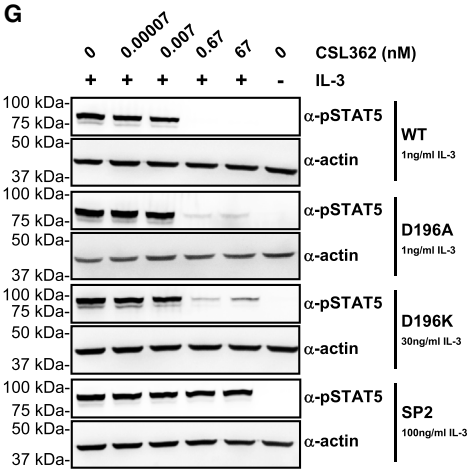


Figure 4. Open-like IL3Rα Mutants Dissociate CSL362 Inhibition of IL-3 Binding from Inhibition of Signaling

(A) Close-up view of the IL3Rα NTD and D2 interface in the open conformation for the WT (upper panel) and modeled D196K mutant (lower panel).

(B) Binding affinity of IL3Rα variants for IL-3, CSL362, or 9F5, measured in saturation binding studies on transfected COS cells. N.B., no binding.

(C) Competition for IL-3 binding to COS cells expressing βc and IL3Rα WT (filled blue circles), SP2 (open blue circles), D196A (filled black circles), D196L (filled purple squares), D196K (filled red squares), or D196R (filled black squares), using labeled IL-3 at the K_D value for each receptor (from B).

(D) Directly measuring IL-3 binding and simultaneous CSL362 binding to COS cells expressing βc and IL3Rα WT (filled and open blue circles), D196A (filled and open black circles), D196L (filled and open purple squares), or D196K (filled and open red squares) using Eu-IL-3 (filled symbols) at the K_D value for each receptor and Tb-CSL362 (open symbols).

(E) Ba/F3 cells expressing βc and the same IL3Rα species in (C) were stimulated with IL-3 and cell proliferation determined.

(F) As for (E), but cells were preincubated with CSL362 followed by IL-3 stimulation at the relevant EC_{50} value (from E).

(G) As for (F), but cells were preincubated with CSL362, stimulated with IL-3 10 min, and lysates immunoblotted for phosphorylated STAT5 or actin. For (C), (D), (E), and (F), the data are the mean of triplicate determinations from a representative experiment (n = 2) and errors represent SD. See also Figure S5.

two models of the IL3 receptor binary complex: one for each of the observed IL3Rα conformations (Figures 3A, 3C, and 3D). The closed IL-3:IL3Rα model (Figure 3A) is analogous to the IL-5:IL5Rα, IL-13:IL13Rα1, and IL-13:IL13Rα2 crystal complexes (Figures 1C, 1D, and S4A). A previous IL-3:IL3Rα model, containing only D2 and D3 of IL3Rα (Dey et al., 2009), and our current IL-3:IL3Rα models guided extensive mutagenesis of IL3Rα and testing for IL-3 binding and signaling. Consistent with the cytokine receptor paradigm, site 1a in IL3Rα utilizes residues in the D2-D3 elbow with F198, N232, E275, and Y278 being major binding determinants, whereas site 1b in the NTD involved residues Q37, Y58, Q69, and G71 (Figure 3). Importantly, many of the IL3Rα site 1 residues identified from our IL-3:IL3Rα models (Figure 3; Table S4) interact with residues in IL-3 that we have previously shown to be functionally important, including S17, D21, T25, R108, F113, K116, and E119 (Barry et al., 1994; Bagley et al., 1996).

Although there is a small functional overlap between the CSL362 and IL-3 epitopes in the IL3Rα NTD, we showed that CSL362 binding and IL-3 function could be dissociated (Figure S4). These results suggested that the overlap between the CSL362 and IL-3 epitopes is not sufficient to fully explain the blocking effect of CSL362. A new mechanism of action for CSL362 was suggested by modeling the IL-3 receptor ternary complex based on the structure of the related GM-CSF receptor complex (Hansen et al., 2008). We previously showed that the functional GM-CSF receptor complex is a dodecamer formed by the association of two hexamers, each containing a βc homodimer with two molecules of ligand and α subunit in a head-to-head configuration (Hansen et al., 2008). This arrangement creates site 4 between hexamers, which can be disturbed by specific antibodies (Hansen et al., 2008; Broxmeyer et al., 2012), and brings βc-associated JAK2 molecules into close proximity to allow receptor transphosphorylation and signaling (Figure 5). Based on the

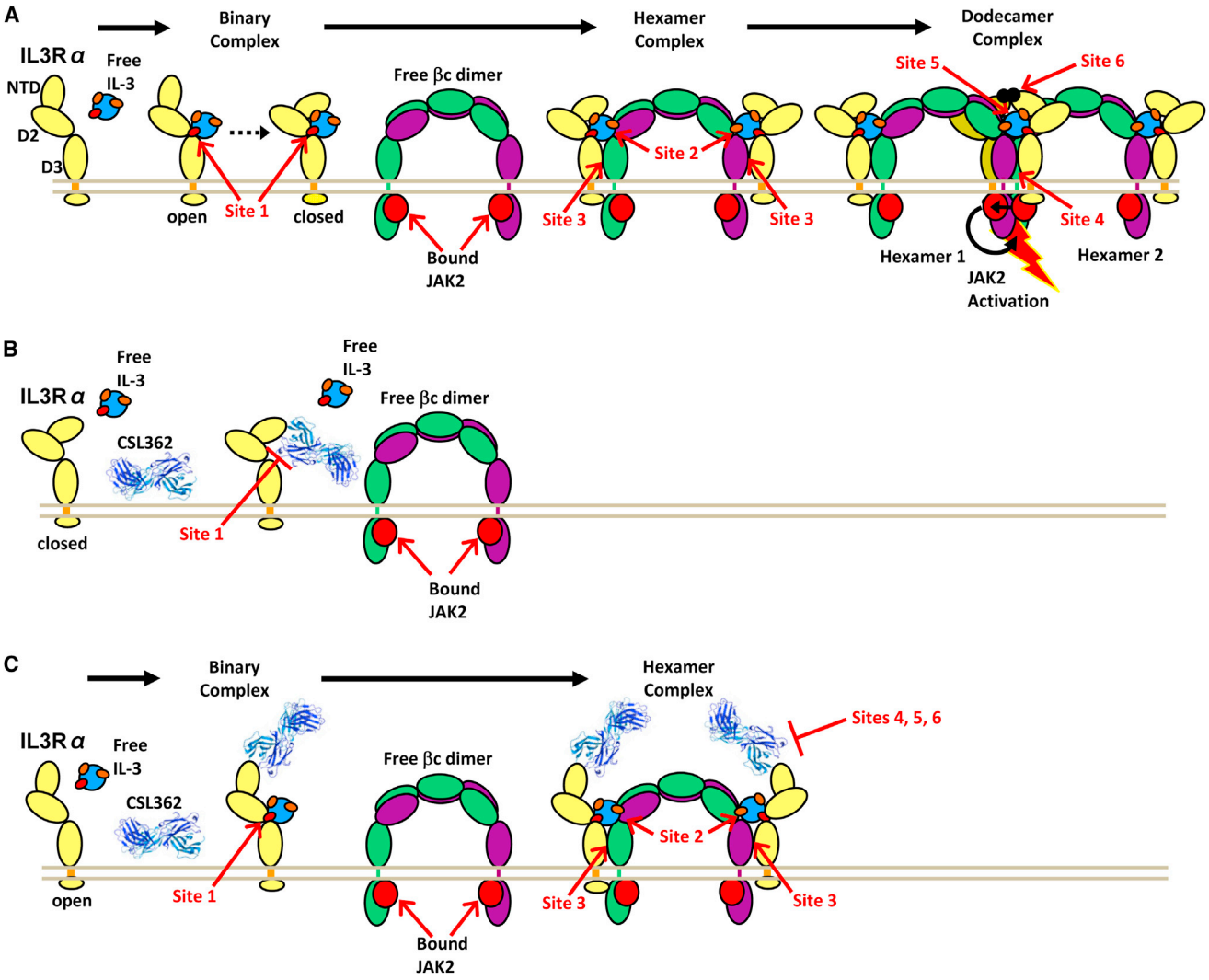


Figure 5. Model of IL-3 Receptor Signaling and Mechanisms of Action of CSL362

(A) Binding of IL-3 to IL3Rα initiates the sequential assembly of the IL-3 receptor into an active signaling complex (dodecamer) through multiple interaction sites. IL3Rα in yellow, IL-3 in blue, the βc homodimer in green and purple, and βc-associated JAK2 in red. Red arrows show interaction sites.

(B) CSL362 binding to closed IL3Rα prevents IL-3 binding to site 1. CSL362 Fab is shown in the blue ribbon.

(C) CSL362 binding to open IL3Rα allows IL-3 binding and hexamer formation but prevents assembly of high-order complexes.

See also Figure S5.

GM-CSF ternary crystal structure, both Patino et al. (2011) and Kusano et al. (2012) demonstrated that IL-5 could form analogous higher-order structures. Our modeling studies indicate that the IL-3 receptor complex can form similar higher-order structures (i.e., hexamers and dodecamers) for both open and closed IL3Rα conformations (Figure S5). Significantly, CSL362 binding to open IL3Rα in these models does not block IL-3 binding or formation of the open hexamer complex (Figures S5E and S5F) but prevents formation of the open dodecamer complex (Figures S5G and S5H).

Residues in the NTD-D2 interface determine the NTD orientation, and through mutagenesis of the S74 (NTD)-interacting residue D196 (D2), we generated IL3Rα mutants that are predicted to adopt an open-like conformation. We showed that cells expressing these mutants bind and respond to IL-3 (Figures 4B and 4E), but IL-3 binding is only partly competed for by CSL362 (Figures 4C and 4D), whereas functional responses to IL-3 are fully competed (Figures 4F and 4G). Mutation of the analogous interacting residues in IL5Rα, D208 or I69 (Figure 1E), results in drastically reduced cytokine binding, suggesting that these residues are critical in maintaining the optimal orientation of the NTD for cytokine binding (Patino et al., 2011). The results support a model of IL-3 receptor activation in which IL3Rα binds IL-3 to form a binary complex through site 1 before recruiting βc through sites 2 and 3 to form a hexameric complex. The recruitment of a second hexamer through sites 4, 5, and 6 (Lopez et al.,

2010; Broughton et al., 2012) forms a dodecameric complex that allows full IL-3 receptor signaling (Figure 5A). This model illustrates the classic mechanism of antibody blockade with CSL362 binding to closed IL3Rα and preventing ligand binding (Figure 5B). The model also highlights a mechanism of antibody blockade whereby CSL362 prevents the formation of the signaling-competent open IL3Rα dodecameric complex (Figure 5C). This model is likely to apply to other members of the βc family and to other cytokine receptor families that exhibit an analogous NTD and highlights a way by which mAbs can exert a "dual hit" to block cytokine receptor signaling.

EXPERIMENTAL PROCEDURES

Generation of Expression Constructs Encoding Full-Length IL3Rα Mutants or Chimeras

Human IL3Rα and GMRα cDNAs were cloned into the pcDNA3.1 expression vector (Invitrogen) and mutations generated by PCR. Selected IL3Rα mutants were cloned in the pSG5 vector (Stratagene) or the pRufPuro:βc-IRES-IL3Rα retroviral expression vector or generated as soluble IL3Rα proteins with a C-terminal 6xHis tag (sIL3Rα-6His). Human βc cDNA was cloned in the pSG5 and pRufPuro:βc-IRES-IL3Rα vectors (details are provided in the Supplemental Experimental Procedures).

Cell-Surface Receptor Binding and Expression Assays

Freestyle 293 suspension cells (Invitrogen) were transfected with plasmid DNA using 293Fectin (Invitrogen) (details are provided in the Supplemental Experimental Procedures). Transfected cells were stained with mAbs 7G3, 9F5 (Sun et al., 1996), and 107D2 (Beckman Coulter Genomics) or with biotinylated human IL-3 (ProSpec-Tany) followed by Streptavidin-PE (BD Biosciences). Functional studies used mAb 7G3 or its humanized variant, mAb CSL362, as they have indistinguishable specificity. mAb 9F5 binds IL3Rα NTD and mAb 107D2 binds IL3Rα D2D3 and were used as controls.

COS cells were electroporated with plasmids encoding IL3Rα alone or in the presence of pSG5H:βc and saturation binding assays with radioiodinated antibody or IL-3 performed (Woodcock et al., 1996). IL-3, 9F5, and CSL362 were radioiodinated with Pierce Pre-Coated Iodination tubes (Thermo Scientific). Dissociation constants were calculated using EBDA and LIGAND programs (Munson and Rodbard, 1980; KELL Radlig, Biosoft). Europium-labeled IL-3 (Eu-IL-3) and terbium-labeled CSL362 (Tb-CSL362) were prepared by DELFIA Systems (PerkinElmer) and used in binding assays as above with cell-bound ligand measured at 615 nm or 545 nm, respectively, using a Victor X5 multiplate reader (PerkinElmer; details are provided in the Supplemental Experimental Procedures).

Surface Plasmon Resonance Binding Assays with sIL3Rα

The binding of IL-3, CSL362, and 9F5 to sIL3Rα was measured using a Biacore 4000 biosensor with a CM-5 sensor chip at 25°C (details are provided in the Supplemental Experimental Procedures).

Functional Studies for the IL-3 Receptor

Ba/F3 cell lines expressing βc and WT or D196 mutant IL3Rα were generated using pRufPuro:βc-IRES-IL3Rα plasmids (details are provided in the Supplemental Experimental Procedures). Cell proliferation assays were performed as previously described (Hansen et al., 2008). For biochemical studies, cells were preincubated with CSL362 for 30 min prior to stimulation with IL-3 for 10 min. Cell lysates were immunoblotted with antibodies specific for phosphorylated STAT5 (Cell Signaling Technology) or actin as a loading control (Millipore).

Modeling of the IL3R Binary and Ternary Complexes

The two IL-3 binary complex models (Figure 3) were constructed using the protein-protein docking algorithm RosettaDock (Lyskov and Gray, 2008). The two distinct IL3Rα crystal structure conformations and a human IL-3 NMR structure (PDB ID: 1JLI model 1; Feng et al., 1996) were aligned via Cα atoms with D3 of GMRα and GM-CSF, respectively, in the GM-CSF ternary complex crystal structure (PDB ID: 3CXE) to produce the rough protein-protein complexes as input to RosettaDock. We used a similar procedure to construct IL-3:IL3Rα:βc models, which were used to generate hexamer and dodecamer IL-3 receptor complexes (Figure S5; details are provided in the Supplemental Experimental Procedures).

ACCESSION NUMBERS

The crystal structure has been deposited to the PDB under accession number 4JZJ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.038.

AUTHOR CONTRIBUTIONS

S.E.B. performed all the crystallographic studies and contributed to structural analyses; T.R.H. and P.S. prepared proteins and complexes for structural and functional studies; T.R.H., M.P.H., B.J.M., M.D., H.H., H.B., E.F.B., W.L.K., and D.H. did binding and functional studies; T.L.N. did molecular modeling; T.L.N. and U.D. assisted with structural analyses; S.J.B., C.M.O., A.D.N., and A.F.L. developed the CSL362 antibody; T.R.H., M.P.H., N.J.W., M.W.P., and A.F.L. designed experiments; S.E.B., T.R.H., M.P.H., and T.L.N. prepared figures; S.E.B., T.R.H., M.P.H., T.L.N., N.J.W., M.W.P., and A.F.L. wrote the paper; and N.J.W., M.W.P., and A.F.L. supervised the research.

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