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Structural basis of signaling blockade by
anti-IL-13 antibody Lebrikizumab

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ABSTRACT

The cytokine interleukin 13 (IL-13) is a major effector molecule for T-helper 2 type (Th2) inflammation and is pathogenic in allergic diseases such as asthma. The effects of IL-13 are mediated via a pathway that is initiated by binding to a heterodimeric receptor consisting of IL-13R α 1 and IL-4R α . Antibodies raised against IL-13 can block its inflammatory effects by interfering with binding to either of the two receptor polypeptides. Lebrikizumab is a monoclonal anti-IL-13 antibody that has shown clinical benefit in a phase II study for the treatment of moderate-to-severe uncontrolled asthma. Here we report the molecular structure of IL-13 in complex with the Fab from Lebrikizumab by X-ray crystallography at 1.9Å resolution. We show that Lebrikizumab inhibits IL-13 signaling by binding to IL-13 with very high affinity and blocking IL-13 binding to IL-4R α . In addition, we use site directed mutations to identify the most important antibody contributors to binding. Our studies define key features of Lebrikizumab binding and its mechanism of action that may contribute to its clinical effects.¹

Keywords:

chronic asthma, allergy, paratope alanine scan, IL-4R α competition

¹ abbreviations: IL-13, interleukin 13. Th2, T-helper type 2. IL-4, interleukin 4. STAT6, signal transducer and activator of transcription 6. Fab, antigen-binding fragment. LebFab, Lebrikizumab Fab. CDR, complementarity determining region. IL-13R α 1, IL-13 receptor alpha 1. IL-4R α , IL-4 receptor alpha. IL-13R α 2, IL-13 receptor alpha 2. rmsd, root-mean-squared deviation. SPR, surface plasmon resonance.

INTRODUCTION

IL-13 is a member of the four-helix bundle short-chain cytokine family that, along with the closely related cytokine IL-4, is associated with allergic type 2 inflammation^{1; 2; 3}. IL-13 binds to two receptors: (1) the heterodimeric combination of IL-13R α 1 and IL-4R α or (2) IL-13R α 2. Of these, the heterodimeric receptor of IL-13R α 1 and IL-4R α is also bound by IL-4.

The affinities of IL-13 and IL-4 for these three receptor polypeptides and the structures of their molecular complexes have been determined previously^{4; 5; 6; 7}. IL-13 binds to IL-13R α 2 with a very high affinity using the combination of two well-separated binding sites, more or less at opposite ends of IL-13 helix-D, to interact with all three domains of the IL-13R α 2 extracellular region⁶. In contrast, IL-13 binds to IL-13R α 1 with a modest affinity and to IL-4R α with a weak affinity, leading to the sequential formation of IL-13/IL-13R α 1 and then IL-13/IL-13R α 1/IL-4R α complexes. The IL-13R α 1-binding site on IL-13 strongly overlaps with the IL-13R α 2-binding site⁶, with differences in detail accounting for the large difference in affinity. In contrast, the IL-4R α -binding site on IL-13 is distinct from those of IL-13R α 1 and IL-13R α 2, on the opposite side of IL-13 and comprised of elements from helices A and C⁵.

IL-13 is produced by many cell types, including Th2 cells, mast cells, basophils, eosinophils, innate type 2 cells, NKT cells, and macrophages; and it promotes several biological processes associated with the pathogenesis of asthma and other allergic diseases^{1; 2; 3}. For instance, IL-13-induced intracellular signal transduction mediated by the IL-13R α 1/IL-4R α receptor, primarily via the phosphorylation of STAT6, is associated with goblet cell hyperplasia and mucus production, B cell class-switching to IgE, induction of chemoattractants for eosinophils and other inflammatory cells, promotion of subepithelial fibrosis, and generation of airway hyperreactivity. In contrast, IL-13R α 2 is considered to

be primarily a decoy receptor, i.e. without a direct signaling function, although under some circumstances IL-13 binding to IL-13R α 2 can promote fibrosis by the induction of TGF β via an intracellular signaling pathway that is poorly understood⁸.

Numerous prior studies have implicated IL-13 and its receptors in the pathogenesis of asthma and allergy^{1; 2; 9; 10}. Polymorphisms of the IL-13 and IL-4R α genes are associated with asthma and allergy, including effects on IgE levels, prevalence of atopy, and the severity of asthmatic disease^{11; 12; 13; 14; 15; 16}. In addition, expression of IL-13 and its receptors is increased in asthma and other allergic diseases, and neutralization or deficiency of IL-13 and its receptors ameliorates disease in preclinical disease models^{17; 18; 19; 20; 21; 22; 23; 24}.

Recently, several monoclonal antibodies against IL-13 have been assessed in clinical studies for the treatment of asthma^{25; 26; 27; 28}. Of these, the anti-IL-13 antibody Lebrikizumab improved lung function in asthmatics who were symptomatic despite treatment with, for the majority, inhaled corticosteroids and a long-acting β_2 -adrenergic receptor-agonist²⁵. The other anti-IL-13 antibodies have shown varying degrees of activity in clinical studies²⁹. The clinical efficacy of anti-IL-13 antibodies may be influenced by the binding affinities of the antibodies to IL-13, as well as the molecular mechanisms by which they block IL-13 signaling. For instance, IL-13 signaling through the IL-13R α 1/IL-4R α receptor can be inhibited by blocking IL-13 binding to either IL-13R α 1 or IL-4R α , but these different molecular mechanisms of blocking IL-13 signaling will have different consequences on the ability of IL-13 to bind to IL-13R α 2.

Although Lebrikizumab has been previously shown to block IL-13 signaling through the IL-13R α 1/IL-4R α receptor²⁵, the binding affinity of Lebrikizumab for IL-13 and the mechanism by which Lebrikizumab blocks IL-13 signaling have not been described in the scientific literature. Here we report the binding affinity

of Lebrikizumab to IL-13 and the crystal structure of the complex between IL-13 and the antigen-binding fragment of Lebrikizumab (LebFab). We find that Lebrikizumab binds with a very high affinity to IL-13 at an epitope that overlaps strongly with the binding site of IL-4R α , and using site-directed mutations of the antibody we identify several key residues in the CDRs of Lebrikizumab that drive this high-affinity binding to IL-13.

RESULTS

Effects of LebFab on IL-13 binding to IL-13-R α 1 and IL-13R α 2

Previous studies have demonstrated that Lebrikizumab blocked IL-13-induced STAT6 phosphorylation and cell proliferation *in vitro* and produced clinical benefits on lung function in asthmatics, both of which are consistent with blockade of the IL-13R α 1/IL-4R α heterodimeric receptor²⁵. In order to determine which receptors are affected by Lebrikizumab binding to IL-13, we performed ELISA assays that show IL-13 binding to neither IL-13R α 1, nor IL-13R α 2, is reduced by the presence of LebFab (Figs 1, S1). These results suggest that Lebrikizumab may block the IL-4R α binding site of IL-13, thereby preventing the formation of an active cytokine/receptor complex consisting of IL-13, IL-13R α 1, and IL-4R α . The intrinsic affinity between IL-13 and IL-4R α alone is too low for direct testing in a similar ELISA assay.

Molecular structure of the LebFab / IL-13 complex by X-ray crystallography

In order to understand the binding site for Lebrikizumab on IL-13, we determined the structure of a complex of LebFab with IL-13 by X-ray crystallography at 1.9Å resolution (Table 1).

The molecular complex shows IL-13 bound by the canonical antigen-recognition site of LebFab. The IL-13 structure is very similar overall to IL-13 from PDB entry 3L5X, another IL-13/Fab complex but wherein IL-13 has a different epitope in contact with the Fab³⁰. The most significant difference in IL-13 C α positions after superpositioning ($\sim 3.8\text{\AA}$) is centered at Pro72 (in the CD loop), which has a water-mediated H-bond with the CDR-L1 Asn30 side chain, but which also experiences a close intermolecular contact in our structure. Absent this and three other short segments with differences up to $\sim 1.9\text{\AA}$, the rmsd for superpositioning of IL-13 C α atoms is $0.74\text{\AA}$, a value which rises to $0.99\text{\AA}$ if all C α atoms are included.

Lebrikizumab is a humanized version of a mouse-derived anti-human IL-13 monoclonal antibody. In our crystals, LebFab displays an elbow angle of 138° . The conformations of the 6 CDR loops (L1, L2, L3 and H1, H2, H3) can be classified³¹ as L1-15-1, L2-8-1, L3-9-cis7-1, H1-13-1, H2-9-1, and H3-anchor-1, respectively, which in each case is the most common configuration for the given CDR length. The fidelity with which these classifications are made is high, with rmsd values for the superpositioning of main chain atoms to the prototype coordinates being less than $0.3\text{\AA}$, except for CDR-H1 where it rises to $0.94\text{\AA}$. The conformational classification of CDR-H3 is restricted to the amino acids in the initial and final polypeptide segments (seven amino acids altogether), and discounts the highly variable intermediate segment for which conformational classes are not a useful concept. There are 12 amino acids in the Lebrikizumab CDR-H3 between Cys92 and Trp103.

The LebFab/IL-13 interface buries $890\text{\AA}^2$ of protein surface from bulk solvent, on each side ($1780\text{\AA}^2$ total). Of the LebFab buried surface, 63% is from the V_H domain and 37% from the V_K domain. At least one residue from each of the 6 CDR loops is within $4\text{\AA}$ of IL-13, and 16 amino acids lose at least $10\text{\AA}^2$ of solvent accessible surface in the IL-13 interaction (Fig. 2)

There are twenty IL-13 residues within 4Å of LebFab, all of them, except Ser32 from the AB loop, being from α -helices B and C and the BC and CD loops. The interface is a mixture of polar and hydrophobic interactions, and includes seven bridging waters that H-bond with both IL-13 and LebFab (Fig 3). The topographic complementarity of the IL-13 and LebFab surfaces is typical of antigen/antibody interactions, with a calculated shape complementarity statistic, S_c , of 0.71³². If the seven bridging water oxygen atoms are considered part of the interface, S_c rises to 0.77. Of the seven aromatic side chains within the interface, all come from LebFab (V κ residues Tyr28, Phe32 and Tyr49 and V $_H$ residues Trp52, Tyr97, Tyr98 and Tyr100) (Fig. 3) and, of these, only V $_H$ residues Tyr98 and Tyr100 provide a polar interaction via their hydroxyl moieties (Fig. 4). IL-13 side chains provide almost exclusively polar interactions, e.g. Ser58, Glu61 and Arg65, while IL-13 residues bearing hydrophobic side chains, e.g. Val54, Ala59 and Ile60, are classified within the epitope by virtue of polar interactions of their main chain atoms, for instance between IL-13 Gly69 and LebFab LC-Tyr28 main chain atoms. Thus, despite a preponderance of aromatic side chains within the LebFab paratope, the main chain atoms of these residues sometimes partake in polar interactions with IL-13 residues. In addition, non-traditional H-bonds between aromatic ring H-atoms and a traditional H-bond acceptor are possible, as between HC-Tyr100 and IL-13 Gln61. The single hydrophobic IL-13 side chain contacting LebFab is Met66, within van der Waals distance of LC-Tyr28 from CDR-L1 (Fig 4).

The intermolecular crystal contacts were inspected for evidence they may have skewed important features described above. The intermolecular environment produces H-bonds and hydrophobic contacts between symmetry-related complexes at several patches (Fig S2). The C $_L$ and C $_H$ 1 domains of LebFab, plus some nearby elements of the V κ and V $_H$ domains, are surrounded by symmetry-related C $_L$ and C $_H$ 1 domains. IL-13, and some nearby elements of the V κ

domain, are surrounded by symmetry-related copies of IL-13. Notably, light chain CDRs -L1 and -L3 both have close contacts with a neighboring copy of IL-13, centered on IL-13 residue Ser36 from the AB loop. The section of CDR-L1 involved in this intermolecular contact does not contribute to the canonical antigen-binding surface of LebFab (see above), but part of CDR-L3 contributes to the canonical paratope while also contacting the symmetry-related IL-13. Since both CDR-L1 and CDR-L3 conform closely to prototype loops, we conclude they do not include artifacts from crystal packing.

Determination of key residues in Lebrikizumab CDR's that drive high-affinity binding to IL-13

Previous studies demonstrated that Lebrikizumab neutralizes the activity of IL-13 with high potency, suggesting that Lebrikizumab binds to IL-13 with very high affinity²⁵. To confirm this, we determined the dissociation constant (K_D) for IL-13 binding to Lebrikizumab by SPR (Biacore). We found that the Biacore-derived K_D for IL-13 binding to wild-type Lebrikizumab is lower than the detection limit, 10 pM. The Lebrikizumab in these experiments was bound to the SPR chip via an anti-Fc reagent. Additional SPR measurements of IL-13 binding to a low density surface of directly amine-coupled Lebrikizumab also revealed kinetic constants at the limit of detection (Fig. S3). In order to understand which Lebrikizumab residues are most vital to this very high affinity interaction, several residues within the structural paratope were mutated one at a time and K_D values for the mutant proteins determined. Conservative mutations of Lebrikizumab residues did not have a measurable effect on K_D , for instance HC-Tyr100 mutated to Phe when measured at 25°C (Table 2). However, mutations to Alanine of six residues had effects on K_D of 5-fold or greater, calculated using 10 pM as the K_D for wild-type Lebrikizumab. These results are illustrated in Figure 4, where some discrepancies between the structural paratope, based on surface

area buried in the complex, and the Biacore binding data are apparent. For instance, HC-Tyr97 protrudes from the LebFab surface and loses more than $75\text{\AA}^2$ of solvent-accessible surface by virtue of its proximity to IL-13 (Fig 2), and yet mutation of this residue to Alanine has an immeasurably small effect on K_D (Table 2 and Fig 4).

Based on affinity measurements of Alanine mutants, the functional paratope resides in a strip of V_H residues lying near the face of IL-13 formed by mostly α -helices B and C (Fig 4). The most important LebFab residues according to the effect of their mutation to Alanine on binding include heavy chain residues Trp52, Asp54, Tyr98 and Tyr100. Asp54 has significant contact with IL-13 (loss of solvent accessible surface, Fig. 3) as it also H-bonds with IL-13's Ser58 (side chain to side chain). Trp52 lacks a polar interaction with IL-13 but has significant contact with IL-13 also centered on Ser58. A measurable but less severe effect arises from mutation of Trp52 to Phe, consistent with a structural role internal to the LebFab which would be much less well filled by Ala. Tyr98 contacts IL-13 residues Arg65 and Ser68 as it also contributes to an H-bonding network with CDR-L1, a water and the main chain of IL-13 Arg65 via its hydroxyl moiety. The contribution of the H-bonding aspect of Tyr98 is not large, as mutation to Phe leaves the dissociation constant beyond the detection limit. Tyr100 contacts IL-13 residues Glu61 and Gln64 and also contributes to an extensive water-mediated H-bonding network that extends to IL-13 residues Val54 and Cys57 in the BC loop and to Gln64. As seen for Tyr98, the H-bonding provided by the Tyr100 hydroxyl makes a marginal contribution to binding, as mutation to Phe causes a measurable but small effect when measured at 37°C (Table 2).

DISCUSSION

Lebrikizumab is a humanized antibody that binds and neutralizes the activity of

IL-13 with high affinity. Clinical studies indicate that Lebrikizumab provides clinical benefit in the treatment of moderate-to-severe uncontrolled asthma²⁵. Here we have determined the structure of a complex between the Lebrikizumab Fab and IL-13, thereby elucidating the structural epitope on IL-13. In addition, we have defined several key residues in the CDR's of Lebrikizumab that are responsible for the high-affinity of Lebrikizumab. The clinical utility of Lebrikizumab derives from its blockade of IL-13 signaling, which we have determined is due to LebFab's interference with IL-13 binding to one of its cell-membrane-bound receptors, IL-4R α , thereby preventing formation of IL-13/IL-13R α 1/IL-14R α signaling complexes. With the X-ray structure reported here, and by reference to previously reported structures of IL-13 with its receptors^{5,6}, we can see precisely how the blockade arises. Figure 5 shows the ternary IL-13/IL-13R α 1/IL-4R α complex⁵, determined using X-ray crystallography. An additional X-ray structure is known, of the complex between IL-13 and the high affinity 'decoy' receptor, IL-13R α 2⁶. There are important differences in detail, but for the purpose of the present discussion we may consider the rough equivalence of IL-13R α 1 and IL-13R α 2 interactions with IL-13 a useful simplification. Also shown in Figure 5 is LebFab from the present work.

There is a quite significant steric overlap between LebFab and IL-4R α when they are both shown as they bind to IL-13. Of the 20 IL-13 residues within 4Å of LebFab, three are also among the 7 residues within 4Å of IL-4R α in the ternary complex (Arg65, Met66 and Gly69) (Fig. 6). We conclude that both these molecules cannot bind IL-13 at the same time. On the other hand, LebFab also seems to be sufficiently distant from IL-13R α 1 that it would not be expected to interfere with IL-13R α 1 (or IL-13R α 2) binding by a direct-contact mechanism (Fig 5). Thus, Lebrikizumab's apparent mechanism of action contrasts with that of another anti-IL-13 antibody called CENTO607, for which a crystal structure has been reported,³³ and which presents an steric conflict for IL-13 interaction with

IL-13R α 1 (and IL-13R α 2) but not for IL-4R α .

The IL-13 and IL-4 affinities for isolated IL-4R α are very different: unmeasurably low⁵ and with a sub-nanomolar K_D ³⁴, respectively. Nonetheless, considering that both IL-13 and IL-4 bind IL-4R α using homologous binding sites, and that a part of this site on IL-13 is also recognized by Lebrikizumab, there is a potential for an interaction between Lebrikizumab and IL-4. We have never observed a measurable effect of Lebrikizumab on IL-4 activity (data not shown).

Several antibodies that bind and neutralize the activity of IL-13 have entered clinical development for the treatment of asthma. These include tralokinumab (CAT-354), IMA-026, IMA-638 and CNTO5825^{25; 26; 27; 28}. While the binding epitope of tralokinumab on IL-13 has not been reported, IMA-026 and CNTO5825 have been reported to bind to the IL-13R α 1/IL-13R α 2 binding site on IL-13^{26; 28}, whereas IMA-638 has been reported to bind to the IL-4R α binding site on IL-13²⁶. Interestingly, IMA-638 had a greater effect on antigen-induced airway response (allergen challenge) in asthmatics than IMA-026²⁶, and it has been suggested that this may be due to better clearance of IL-13 in patients treated with IMA-638 arising from IL-13/IMA-638 complexes, but not IL-13/IMA-026 complexes, being scavenged by the decoy receptor IL-13R α 2³⁵. Alternatively, differences in binding affinities of IMA-638 and IMA-026 may account for differences in the activity of these antibodies in allergen challenge studies. However, IMA-638 showed no effect on symptoms, lung function, or time to exacerbation compared to placebo in patients with uncontrolled asthma who were also receiving inhaled corticosteroids (ICS) or ICS plus long-acting β 2-agonists²⁶. The K_D of Lebrikizumab binding to IL-13 is < 10 pM as determined by Biacore in our studies, tighter than has been reported previously for other anti-IL-13 antibodies (165 - 14 pM^{36; 37}). The clinical effects of Lebrikizumab among treated asthmatics, such as they may differ from those from other anti-IL-13 antibodies, may be distinguished by its very high affinity, the exact details of its

epitope on IL-13, the interference of IL-4R α binding, and the potential for IL-13 to be scavenged via IL-13R α 2 even when bound by Lebrikizumab, or by combinations of these characteristics.

MATERIALS AND METHODS

Protein complex formation and crystal structure determination

For Fab'2 generation, 125 mg Lebrikizumab was adjusted to pH 4.2 with 0.5 M sodium acetate buffer. Pepsin was added to a final concentration of 1% w/w of the IgG. The mixture was incubated at 37°C for 1 hour. The protein was diluted 3 fold with water and loaded onto a SPHP (GE Healthcare) 5 mL column equilibrated in 25 mM sodium acetate pH 5.0. The column was washed with starting buffer (25 mM sodium acetate pH 5.0) and eluted with 20-25 column volumes with salt gradient to 0.3 M NaCl. Peaks were assessed by mass spectrometry to identify the Fab'2 peak pool. Preparation of Fab' was accomplished by adjusting the pH of the Fab'2 pool to 7.4, then adding 1mM DTT and incubating 2 hours at 20 °C. Reduction was assessed by mass spectrometry. Addition of 3 mM dehydroascorbic acid solution (100 mM in dimethylacetamide) was mixed and incubated 2 hrs at 20°C. Mass spectrometry was used to assay for complete oxidation of the heavy chain/light chain intrachain disulfide bridge. The Fab' was further purified by size exclusion using S200 (GE Healthcare) in phosphate-buffered saline. Recombinant human IL-13 was purchased from R&D Systems (Minneapolis, MN). The complex was prepared and purified as reported³³, except that glycerol concentration was reduced from 10% to 2%.

Crystallization of the complex was carried out by vapor diffusion in sitting drops at 18°C. The experiments consisted of 30 nL protein complex plus 30 nL reservoir using an Echo Acoustic Liquid Handler (Labcyte). Initial screens were performed

using PEG and PEGII screens (Qiagen) and PEGRx (Hampton). Monoclinic crystals of the complex appeared after 2 days from a PEGRx experiment using 30% w/v PEG1000, 0.1M sodium malonate pH 8.0, 0.1M TRIS pH 8.0. After one week, crystals were harvested directly from the screening tray and preserved by immersion in liquid nitrogen after a brief treatment in pure reservoir.

Diffraction data extending to 1.90 Å were collected at synchrotron beamline 5.0.2 at the Advanced Light Source and reduced^{38;39} in space group C2. The structure was solved by molecular replacement⁴⁰ based on an expectation of a single complex in the crystallographic asymmetric unit according to a V_M of 2.5 Å³/Dalton and using IL-13 from pdb entry 3L5X and a Fab variable region (with CDR loops removed) and constant region from PDB entries 1FVC and 1FVD, respectively. Model building and manipulation⁴¹ and refinement^{42; 43; 44} led to the final model with agreement factors $R=0.179$ and $R_{FREE}=0.219$. See Table 1.

ELISA assays for effect of LebFab on IL-13 binding to receptors

In a 96-well plate, a 200 µg/mL (4.44 µM) solution of Lebrikizumab Fab was serially diluted two-fold in assay buffer (phosphate-buffered saline (PBS), pH 7.5, containing 0.05% Tween 20 and 0.5% bovine serum albumin (BSA)) to provide a concentration range for 0.10 to 200 µg/mL (2.2 nM to 4.44 µM). The volume of each dilution was 35 µL. Biotinylation of IL-13 was accomplished using the Thermo EZ-Link Sulfo-NHS-LC-Biotin Kit (Pierce Biotechnology) according to the manufacturer's protocol using a 20-fold molar excess. To each well, 35 µL of a 120 nM solution of biotin-IL-13 was added. The mixture was incubated for 40 minutes at room temperature. Following incubation, the contents of the well were transferred to a 96-well Nunc Maxisorp plate (Roskilde, Denmark) that was coated overnight with 50 µL of a 2.0 µg/mL solution of soluble IL-13Rα1-Fc protein in PBS and then blocked with PBS containing 1% BSA. After a 40-minute

incubation, the plate was washed four times in wash buffer (PBS containing 0.05% Tween 20). Each well then received 50 μ L of a streptavidin-horseradish peroxidase solution (Caltag Laboratories, Invitrogen; Carlsbad, CA) and was incubated for 40 minutes. Following four washes with wash buffer, 50 μ L of tetramethylbenzidine (TMB) substrate (KPL; Gaithersburg, MD) was added to each well. After several minutes, 50 μ L of a 1 N solution of HCL was added to stop the reaction. The plate was read at 450 nm using a Spectra Max 340 plate reader (Molecular Devices; Sunnyvale, CA). For each sample, the optical density reading at 450 nm was plotted against concentration. Curves were plotted in Kaleidagraph (Synergy Software; Reading, PA) and fitted using a 4-parameter fit or plotted point-to-point. The same procedures were followed in an assay using IL-13R α 2, except that LebFab was used at concentrations approximately 10-fold lower (0.01 to 20 μ g/mL (0.1 nM to 0.44 μ M), and the concentration of biotin-IL-13 was reduced to 0.8 nM. A different anti-IL-13 antibody (11H4v6) was shown able to completely eliminate IL-13 binding to both IL-13R α 1 and IL-13R α 2, and on this basis it was employed as an experimental control.

Site-directed mutation of Lebrikizumab

Mutations were introduced into the heavy and light chains of Lebrikizumab by site-directed Kunkel mutagenesis⁴⁵. The plasmid DNA containing the heavy or light chain mutation clones were transfected into 293s cells and harvested seven days later. IgG was isolated from the harvest media by Protein A affinity chromatography.

Binding assay for Lebrikizumab variants using surface plasmon resonance

The binding kinetics of the Lebrikizumab variants were measured using surface

plasmon resonance on a Biacore 3000 instrument (GE Healthcare). Anti-human Fc (GE Healthcare) was immobilized on a CM5 sensor chip via amine-based coupling using the manufacturer-provided protocol. A high density anti-human Fc surface (>5000 resonance units (RU)) was employed in order to minimize the contribution to the observed antigen-binding kinetics from dissociation of the antibody from the capture surface. The Lebrikizumab variants were captured at a level of 600 RU. Sensorgrams from passage of human IL-13 at concentrations of 0, 3.13, 6.25, 12.50, 25.0, and 50.0 nM were recorded using an injection time of 2 minutes with a flow rate of 30 μ L/min, at temperatures of 25°C and 37°C, and with a running buffer of 10 mM HEPES, pH 7.4, 150 mM NaCl, and 0.005% Tween 20. After injection, disassociation of the cytokine was monitored for 900 seconds in running buffer. The surface was regenerated between binding cycles with a 50 μ L injection of 3 M magnesium chloride. The primary sensorgrams were corrected using a blank cell run in parallel and by zero-point adjustment using the 0 nM IL-13 buffer. After corrections, sensorgrams were analyzed using a 1:1 Langmuir binding model with software supplied by the manufacturer to calculate the kinetic and binding constants.

Accession: Final coordinates and structure factors for the LebFab/IL-13 complex have been deposited at <http://www.rcsb.org> under accession code 4I77.

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References

1. Hershey, G. K. (2003). IL-13 receptors and signaling pathways: an evolving web. *J Allergy Clin Immunol* **111**, 677-690.
2. Wills-Karp, M. (2004). Interleukin-13 in asthma pathogenesis. *Immunol Rev* **202**, 175-190.
3. Wynn, T. A. (2003). IL-13 effector functions. *Annu Rev Immunol* **21**, 425-456.
4. Hage, T., Sebald, W. & Reinemer, P. (1999). Crystal structure of the interleukin-4/receptor alpha chain complex reveals a mosaic binding interface. *Cell* **97**, 271-281.
5. LaPorte, S. L., Juo, Z. S., Vaclavikova, J., Colf, L. A., Qi, X., Heller, N. M., Keegan, A. D. & Garcia, K. C. (2008). Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell* **132**, 259-272.
6. Lupardus, P. J., Birnbaum, M. E. & Garcia, K. C. (2010). Molecular basis for shared cytokine recognition revealed in the structure of an unusually high affinity complex between IL-13 and IL-13Ralpha2. *Structure* **18**, 332-342.
7. Mueller, T. D., Zhang, J. L., Sebald, W. & Duschl, A. (2002). Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochim Biophys Acta* **1592**, 237-250.
8. Fichtner-Feigl, S., Strober, W., Kawakami, K., Puri, R. K. & Kitani, A. (2006). IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* **12**, 99-106.
9. Brightling, C. E., Saha, S. & Hollins, F. (2010). Interleukin-13: prospects for new treatments. *Clin Exp Allergy* **40**, 42-29.
10. Finkelman, F. D., Hogan, S. P., Hershey, G. K., Rothenberg, M. E. & Wills-Karp, M. (2010). Importance of cytokines in murine allergic airway disease and human asthma. *J Immunol* **184**, 1663-1674.
11. Graves, P. E., Kabesch, M., Halonen, M., Holberg, C. J., Baldini, M., Frittsch, C., Weiland, S. K., Erickson, R. P., von Mutius, E. & Martinez, F. D. (2000). A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. *J Allergy Clin Immunol* **105**, 506-513.

12. Heinzmann, A., Mao, X. Q., Akaiwa, M., Kreomer, R. T., Gao, P. S., Ohshima, K., Umeshita, R., Abe, Y., Braun, S., Yamashita, T., Roberts, M. H., Sugimoto, R., Arima, K., Arinobu, Y., Yu, B., Kruse, S., Enomoto, T., Dake, Y., Kawai, M., Shimazu, S., Sasaki, S., Adra, C. N., Kitaichi, M., Inoue, H., Yamauchi, K., Tomichi, N., Kurimoto, F., Hamasaki, N., Hopkin, J. M., Izuhara, K., Shirakawa, T. & Deichmann, K. A. (2000). Genetic variants of IL-13 signalling and human asthma and atopy. *Hum Mol Genet* **9**, 549-559.
13. Howard, T. D., Whittaker, P. A., Zaiman, A. L., Koppelman, G. H., Xu, J., Hanley, M. T., Meyers, D. A., Postma, D. S. & Bleecker, E. R. (2001). Identification and association of polymorphisms in the interleukin-13 gene with asthma and atopy in a Dutch population. *Am J Respir Cell Mol Biol* **25**, 377-384.
14. van der Pouw Kraan, T. C., van Veen, A., Boeije, L. C., van Tuyl, S. A., de Groot, E. R., Stapel, S. O., Bakker, A., Verweij, C. L., Aarden, L. A. & van der Zee, J. S. (1999). An IL-13 promoter polymorphism associated with increased risk of allergic asthma. *Genes Immun* **1**, 61-65.
15. Vercelli, D. (2008). Advances in asthma and allergy genetics in 2007. *J Allergy Clin Immunol* **122**, 267-271.
16. Wenzel, S. E., Balzar, S., Ampleford, E., Hawkins, G. A., Busse, W. W., Calhoun, W. J., Castro, M., Chung, K. F., Erzurum, S., Gaston, B., Israel, E., Teague, W. G., Curran-Everett, D., Meyers, D. A. & Bleecker, E. R. (2007). IL4R alpha mutations are associated with asthma exacerbations and mast cell/IgE expression. *Am J Respir Crit Care Med* **175**, 570-576.
17. Calderon, C., Rivera, L., Hutchinson, P., Dagher, H., Villanueva, E., Ghildyal, R., Bardin, P. G. & Freezer, N. J. (2009). T-cell cytokine profiles are altered in childhood asthma exacerbation. *Respirology* **14**, 264-269.
18. Grunig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M. & Corry, D. B. (1998). Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261-2263.
19. Humbert, M., Durham, S. R., Kimmitt, P., Powell, N., Assoufi, B., Pfister, R., Menz, G., Kay, A. B. & Corrigan, C. J. (1997). Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin Immunol* **99**, 657-665.
20. Lee, Y. C., Lee, K. H., Lee, H. B. & Rhee, Y. K. (2001). Serum levels of

- interleukins (IL)-4, IL-5, IL-13, and interferon-gamma in acute asthma. *J Asthma* **38**, 665-671.
21. Saha, S. K., Berry, M. A., Parker, D., Siddiqui, S., Morgan, A., May, R., Monk, P., Bradding, P., Wardlaw, A. J., Pavord, I. D. & Brightling, C. E. (2008). Increased sputum and bronchial biopsy IL-13 expression in severe asthma. *J Allergy Clin Immunol* **121**, 685-691.
 22. Subrata, L. S., Bizzantino, J., Mamessier, E., Bosco, A., McKenna, K. L., Wikstrom, M. E., Goldblatt, J., Sly, P. D., Hales, B. J., Thomas, W. R., Laing, I. A., LeSouef, P. N. & Holt, P. G. (2009). Interactions between innate antiviral and atopic immunoinflammatory pathways precipitate and sustain asthma exacerbations in children. *J Immunol* **183**, 2793-2800.
 23. Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T. Y., Karp, C. L. & Donaldson, D. D. (1998). Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261.
 24. Cohn, L., Homer, R. J., MacLeod, H., Mohrs, M., Brombacher, F. & Bottomly, K. (1999). Th2-induced airway mucus production is dependent on IL-4R α , but not on eosinophils. *J Immunol* **162**, 6178-6183.
 25. Corren, J., Lemanske, R. F., Hanania, N. A., Korenblat, P. E., Parsey, M. V., Arron, J. R., Harris, J. M., Scheerens, H., Wu, L. C., Su, Z., Mosesova, S., Eisner, M. D., Bohen, S. P. & Matthews, J. G. (2011). Lebrikizumab treatment in adults with asthma. *N Engl J Med* **365**, 1088-1098.
 26. Gauvreau, G. M., Boulet, L. P., Cockcroft, D. W., Fitzgerald, J. M., Carlsten, C., Davis, B. E., Deschesnes, F., Duong, M., Durn, B. L., Howie, K. J., Hui, L., Kasaian, M. T., Killian, K. J., Strinich, T. X., Watson, R. M., Y, N., Zhou, S., Raible, D. & O'Byrne, P. M. (2011). Effects of interleukin-13 blockade on allergen-induced airway responses in mild atopic asthma. *Am J Respir Crit Care Med* **183**, 1007-1014.
 27. Piper, E., Brightling, C., Niven, R., Oh, C., Faggioni, R., Poon, K., She, D., Kell, C., May, R. D., Geba, G. P. & Molfino, N. A. A phase 2 placebo-controlled study of tralokinumab in moderate-to-severe asthma. *Eur Respir J* : 10.1183/09031936.00223411
 28. van Hartingsveldt, B., Nnane, I. P., Bouman-Thio, E., Loza, M. J., Piantone, A., Davis, H. M. & Petty, K. J. (2012). Safety, tolerability and pharmacokinetics of a human anti-IL-13 monoclonal antibody (CNTO 5825) in an ascending single-dose first-in-human study. *Br J Clin*

Pharmacol 10.1111/j.1365-2125.2012.04477.x.

29. Ingram, J. L. & Kraft, M. (2012). IL-13 in asthma and allergic disease: Asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* **130**, 829-842.
30. Fransson, J., Teplyakov, A., Raghunathan, G., Chi, E., Cordier, W., Dinh, T., Feng, Y., Giles-Komar, J., Gilliland, G., Lollo, B., Malia, T. J., Nishioka, W., Obmolova, G., Zhao, S., Zhao, Y., Swanson, R. V. & Almagro, J. C. (2010). Human framework adaptation of a mouse anti-human IL-13 antibody. *J Mol Biol* **398**, 214-231.
31. North, B., Lehmann, A. & Dunbrack, R. L., Jr. (2011). A new clustering of antibody CDR loop conformations. *J Mol Biol* **406**, 228-256.
32. Lawrence, M. C. & Colman, P. M. (1993). Shape complementarity at protein/protein interfaces. *J Mol Biol* **234**, 946-950.
33. Teplyakov, A., Obmolova, G., Wu, S. J., Luo, J., Kang, J., O'Neil, K. & Gilliland, G. L. (2009). Epitope mapping of anti-interleukin-13 neutralizing antibody CNTO607. *J Mol Biol* **389**, 115-123.
34. Andrews, A. L., Holloway, J. W., Holgate, S. T. & Davies, D. E. (2006). IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets. *J Immunol* **176**, 7456-7461.
35. Kasaian, M. T., Raible, D., Marquette, K., Cook, T. A., Zhou, S., Tan, X. Y. & Tchistiakova, L. (2011). IL-13 antibodies influence IL-13 clearance in humans by modulating scavenger activity of IL-13Ralpha2. *J Immunol*, 561-569.
36. Kasaian, M. T., Tan, X. Y., Jin, M., Fitz, L., Marquette, K., Wood, N., Cook, T. A., Lee, J., Widom, A., Agostinelli, R., Bree, A., Schlerman, F. J., Olland, S., Wadanoli, M., Sypek, J., Gill, D., Goldman, S. J. & Tchistiakova, L. (2008). Interleukin-13 neutralization by two distinct receptor blocking mechanisms reduces immunoglobulin E responses and lung inflammation in cynomolgus monkeys. *J Pharmacol Exp Ther* **325**, 882-892.
37. May, R. D., Monk, P. D., Cohen, E. S., Manuel, D., Dempsey, F., Davis, N. H., Dodd, A. J., Corkill, D. J., Woods, J., Joberty-Candotti, C., Conroy, L. A., Koentgen, F., Martin, E. C., Wilson, R., Brennan, N., Powell, J. & Anderson, I. K. (2012). Preclinical development of CAT-354, an IL-13 neutralizing antibody, for the treatment of severe uncontrolled asthma. *Br J Pharmacol* **166**, 177-193.

38. Otwinowski, Z. & Minor, W. (1996). Processing of X-ray diffraction data collected in oscillation mode. *Methods in Enzymol* **276**, 307-326.
39. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A. & Wilson, K. S. (2011). Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* **67**, 235-242.
40. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658-674.
41. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501.
42. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C. & Zwart, P. H. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-21.
43. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* **53**, 240-255.
44. Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., Roversi, P., Sharff, A., Smart, O. S., Vonrhein, C. & Womack, T. O. (2011). BUSTER version 2.11.2. Global Phasing Ltd., Cambridge, United Kingdom.
45. Kelley, R. F. & Meng, Y. G. (2012). Methods to engineer and identify IgG1 variants with improved FcRn binding or effector function. In *Methods Mol Biol: Antibody Methods and Protocols* (Proetzel, G. & Ebersbach, H., eds.), Vol. 901, pp. 277-293. Humana Press, New York.

Figure Captions

Figure 1. Inhibition of IL-13 binding to receptor. ELISA was used to determine if the Fab of Lebrikizumab (●) would reduce binding of cytokine to

receptor. (A) Lebrikizumab Fab does not inhibit binding of biotinylated human IL-13 (60 nM) to human IL-13R α 1. Control anti-IL-13 human IgG (■) reduces binding of biotinylated human IL-13 from a max level of cytokine binding (▼) to a level of no cytokine (▲). (B) Lebrikizumab Fab does not inhibit binding of biotinylated human IL-13 (0.4 nM) to human IL-13R α 2. Error bars represent $\pm$ standard deviation of three independent measurements.

Figure 2. The LebFab structural paratope. V_H dark blue, V_K light blue, with CDR residues colored according to the magnitude of solvent accessible surface lost in the IL-13 interaction (red >75Å², orange 20-75Å², yellow 10-20Å²).

Figure 3. The LebFab/IL-13 interface. Rotated about 90° from Figure 2, the LebFab side chains from Figure 2 are illustrated, plus those within H-bonding distance to an interfacial water. Prominent LebFab residues are labelled using a single-letter amino acid code. IL-13 is green, with side chains included for residues within 4Å of LebFab. Black spheres are water atoms bridging between LebFab and IL-13. Dotted lines are distances between polar atoms up to 3.2Å.

Figure 4. Functional paratope of Lebrikizumab determined by Alanine mutations and Biacore K_D determinations. Light chain (LC) in cyan, heavy chain (HC) in blue and semi-transparent IL-13 helices B and C outlined in black. Orange are residues with at least a 47-fold effect on K_D, yellow are at least a 5-fold effect, and the white unchanged from wild-type.

Figure 5. LebFab prevents binding of IL-13 to receptor IL-4R α . The ternary complex between IL-13, IL-13R α 1 and IL-4R α is shown with the LebFab/IL-13 complex after superpositioning on IL-13. Part of IL-4R α seems to be "inside" LebFab, an impossibility. The wire frame represents Fab CNT0607, from pdb entry 3G6D. IL-13 α -helices are labeled.

Figure 6. LebFab epitope and IL-4R α binding site on IL-13 overlap. The 4Å contact zones from LebFab (left, in red) and IL-4R α (right, in magenta) clearly share IL-13 residues in common. The view of each IL-13 is from the same direction after superpositioning. The inexact congruence between the overall IL-13 shapes arises from differences in residues missing due to disorder in the refined structures.

Table 1. X-ray Data Collection and Refinement for LebFab/IL-13

<i>Data collection</i>	ALS 5.0.2
X-ray wavelength (Å)	1.0
space group	C2
unit cell edges (Å)	<i>a</i> =83.72, <i>b</i> = 64.77, <i>c</i> = 117.1
unit cell angles (°)	α = 90, β = 106.1, γ = 90
<i>V_M</i> (Å ³ /Dalton)	2.5
Resolution (Å)	30 – 1.90 (1.97 – 1.90)
Rsym ^{a,b}	0.065 (0.517)
Number of observations	171802
Unique reflections	46433
Completeness (%) ^b	98.3 (92.1)
<i>I</i> / σ <i>I</i> ^b	18.1 (2.0)
Wilson B (Å ²)	29
<i>Refinement</i>	
Resolution (Å)	30 - 1.9
Refs (<i>F</i> >0 σ (<i>F</i>))	45487
<i>R_{WORK}</i> ^c , <i>R_{FREE}</i>	0.179, 0.219
Molecules/asymmetric unit	1
protein residues	534
solvent molecules	379
atoms ^d	4472 (51)
Mean B-factor (Å ²)	49
Rmsd bonds (Å)	0.010
Rmsd angles (°)	1.2
Number of TLS groups	9
Ramachandran (%)	96/3/1

^a $R_{sym} = \sum |I| - \langle I \rangle / \sum \langle I \rangle$, where *I* is the intensity of a single observation and $\langle I \rangle$ the average intensity for symmetry equivalent observations.

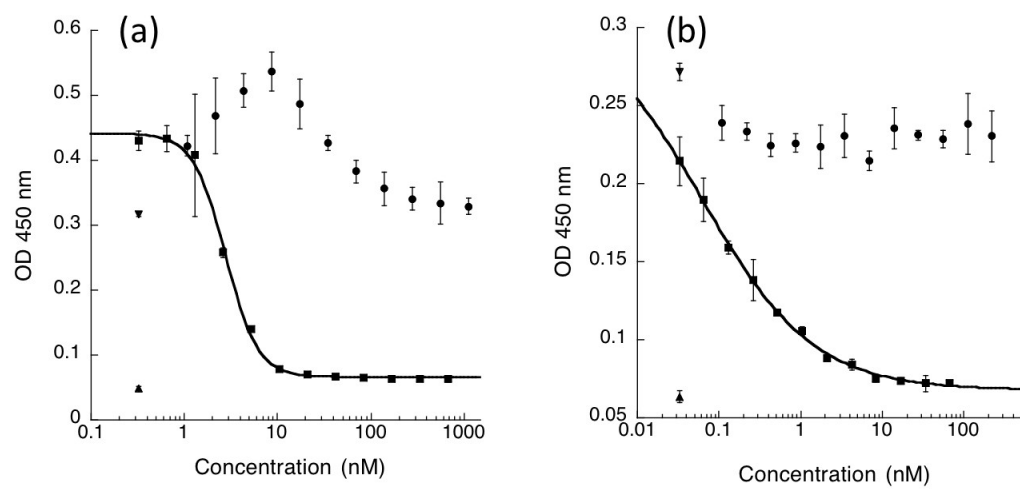
^b In parenthesis, for the highest resolution shell.

^c $R_{WORK} = \sum |F_o - F_c| / \sum |F_o|$, where *F_o* and *F_c* are observed and calculated structure factor amplitudes, respectively. *R_{FREE}* is calculated as *R_{WORK}* for reflections sequestered from refinement.

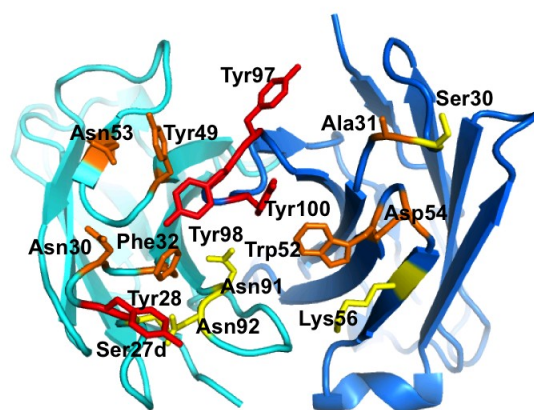
^d In parenthesis, the number of atoms assigned less than unit occupancy.

Table 2. Affinities of Lebrikizumab mutants for IL-13 by Biacore

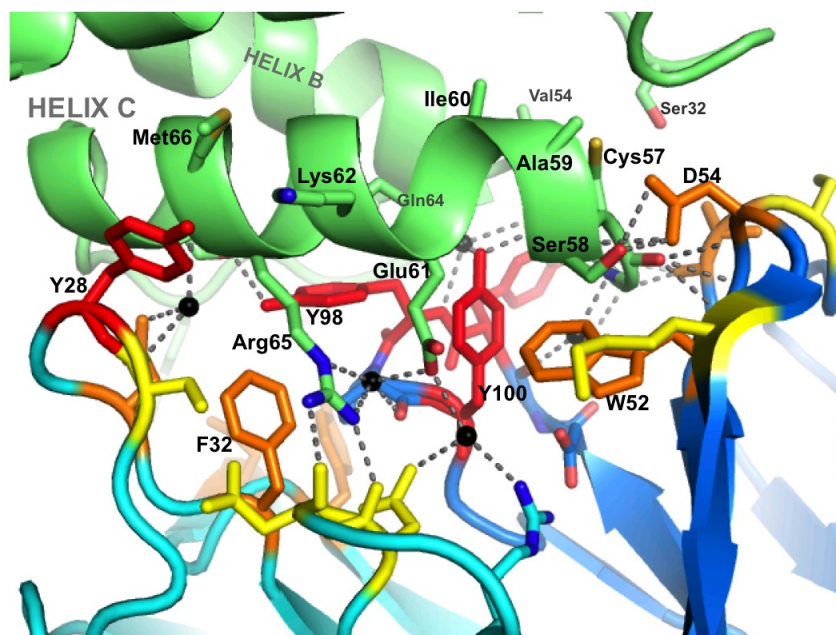
mutatio n	K_D (25°C) (nM)	K_D/.01 0	K_D (37°C) (nM)	K_D/.01 0
HC-Y97F	<0.010		<0.010	
HC-Y97A	<0.010		<0.010	1
HC-Y98F	<0.010		<0.010	
HC-Y98A	0.049 ±0.005	5	0.466 ±0.075	47
HC-Y100F	<0.010		0.029 ±0.016	
HC-Y100A	0.174 ±0.062	17	0.805 ±0.117	80
HC-D54A	0.361 ±0.112	36	1.567 ±0.345	157
HC-W52F	0.346 ±0.098		1.173 ±0.231	
HC-W52A	0.355 ±0.117	36	1.357 ±0.151	135
LC-Y28F	<0.010		<0.010	
LC-Y28A	<0.010	1	0.141 ±0.017	14
LC-N91A	0.183 ±0.058	18	0.054 ±0.018	5
WT	<0.010		<0.010	



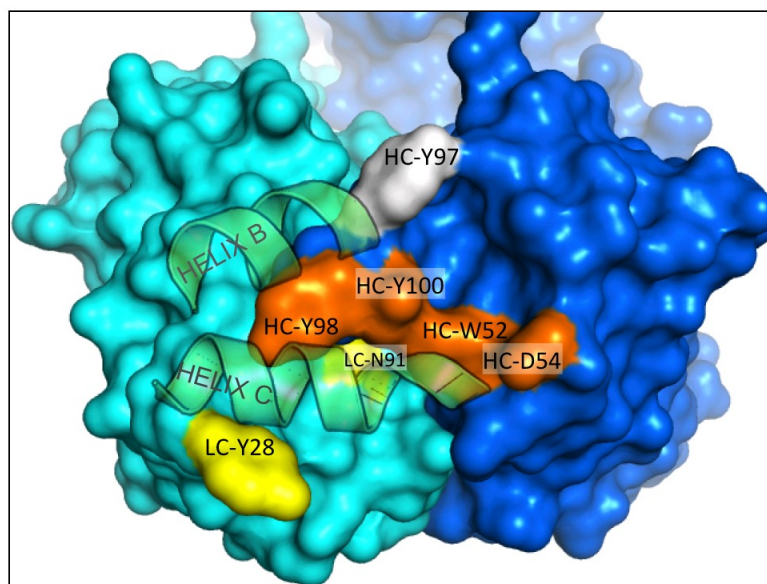
Ultsch et al. Figure 1



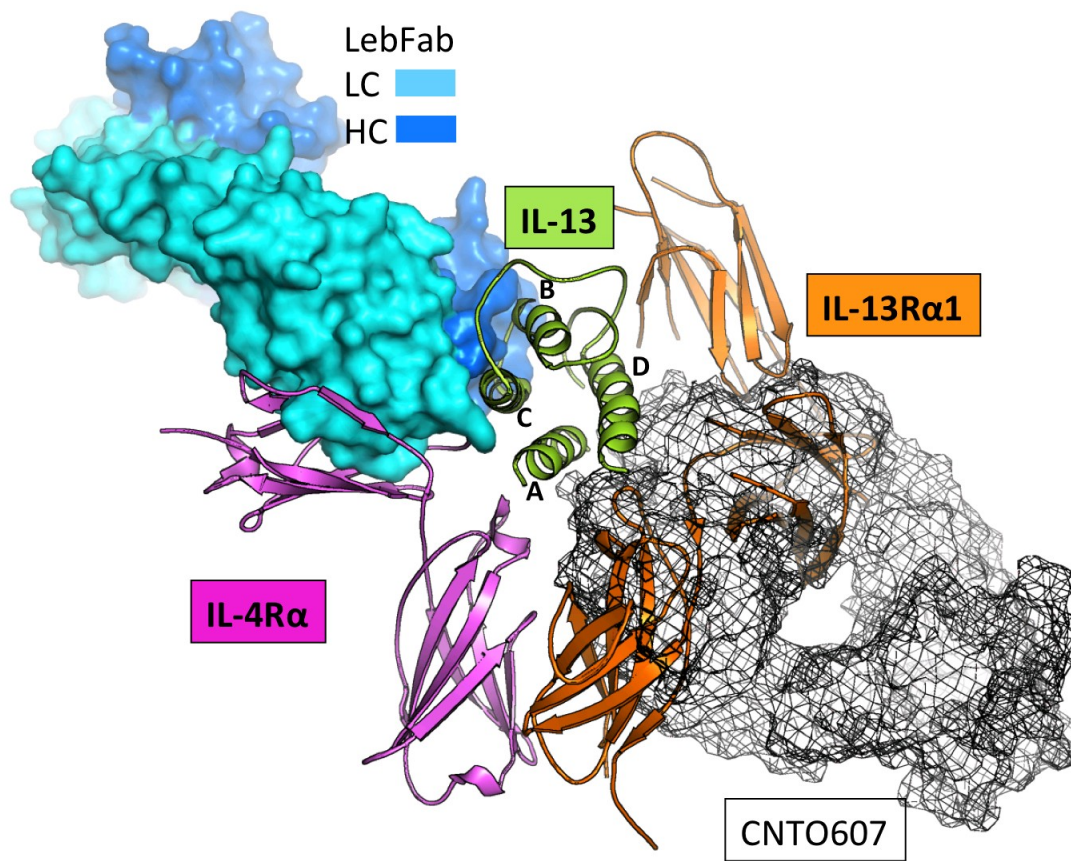
Ultsch et al. Figure 2



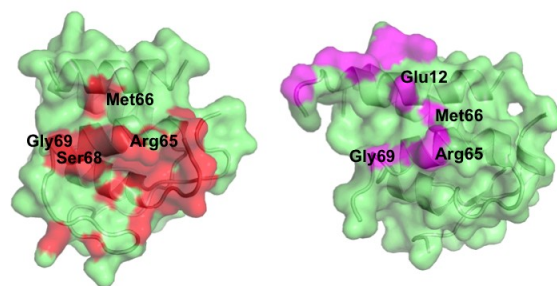
Ultsch et al. Figure 3



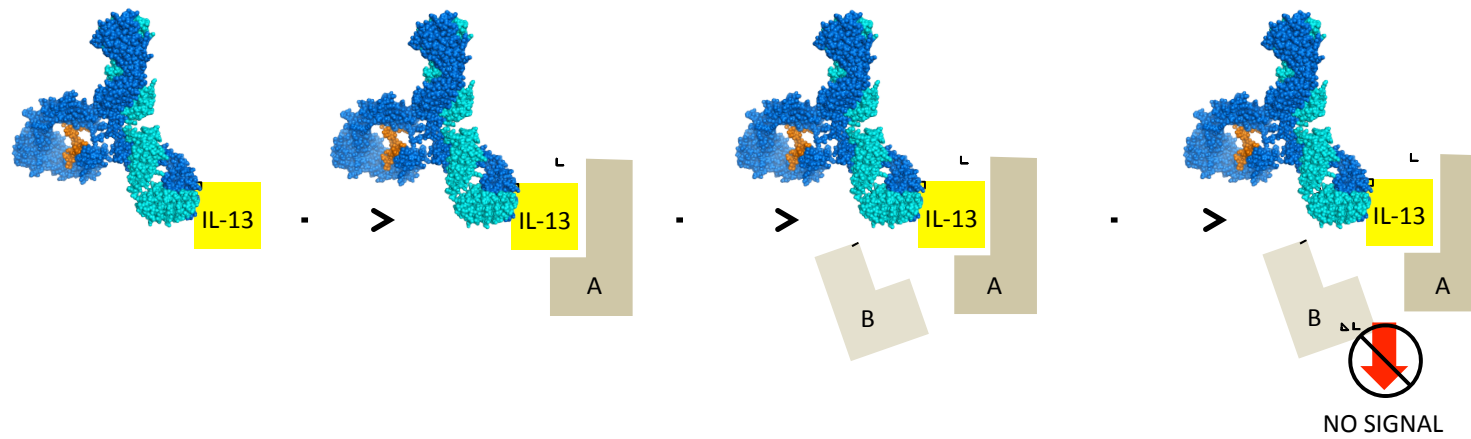
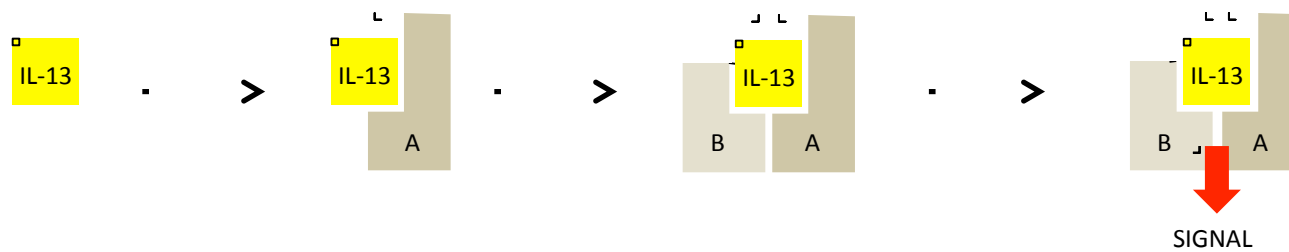
Ultsch et al. Figure 4



Ultsch et al. Figure 5



Ultsch et al. Figure 6



Highlights

The cytokine interleukin 13 is a major immune effector associated with asthma.

Anti-IL-13 Lebrikizumab has a dissociation constant less than 10 picomolar.

Lebrikizumab prevents binding of IL-4Ra, a receptor required for signaling.