Structure of a Potential Therapeutic Antibody Bound to Interleukin-16 (IL-16)

MECHANISTIC INSIGHTS AND NEW THERAPEUTIC OPPORTUNITIES

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Interleukin-16 (IL-16) is reported to be a chemoattractant cytokine and modulator of T-cell activation, and has been proposed as a ligand for the co-receptor CD4. The secreted active form of IL-16 has been detected at sites of T cell-mediated inflammation, such as those seen in autoimmune diseases, ischemic reperfusion injury (IRI), and tissue transplant rejection. Neutralization of IL-16 recruitment to its receptor, using an anti-IL-16 antibody, has been shown to significantly attenuate inflammation and disease pathology in IRI, as well as in some autoimmune diseases. The 14.1 antibody is a monoclonal anti-IL-16 antibody, which when incubated with CD4+ cells is reported to cause a reduction in the Tn1-type inflammatory response. Secreted IL-16 contains a characteristic PDZ domain. PDZ domains are typically characterized by a defined globular structure, along with a peptide-binding site located in a groove between the αβ and ββ structural elements and a highly conserved carboxylate-binding loop. In contrast to other reported PDZ domains, the solution structure previously reported for IL-16 reveals a tryptophan residue obscuring the recognition groove. We have solved the structure of the 14.1 Fab fragment in complex with IL-16, revealing that binding of the antibody requires a conformational change in the IL-16 PDZ domain. This involves the rotation of the αβ-helix, accompanied movement of the peptide groove obscuring tryptophan residue, and consequent opening up of the binding site for interaction. Our study reveals a surprising mechanism of action for the antibody and identifies new opportunities for the development of IL-16-targeted therapeutics, including small molecules that mimic the interaction of the antibody.

Intercellular (IL-16) is also known as lymphocyte chemoattractant factor) was first described in 1982 as a T-cell chemoattractant factor produced by antigen and mitogen-stimulated lymphocytes (1). An array of immune and non-immune cells are now known to express IL-16 as one aspect of an inflammatory response, including CD4+ and CD8+ T cells, eosinophils, monocytes, mast cells, and dendritic cells (2–5). In addition, IL-16 has been reported to promote the entry of resting CD4+ T cells into the cell cycle, and the up-regulation of IL-2 receptor and major histocompatibility (MHC) class II proteins on cell surfaces (6, 7).

Human IL-16 (hIL-16) is expressed as a 631-amino acid precursor protein and contains three PDZ domains, along with an N-terminal CcN motif, encompassing both CK2 and cdc2 kinase phosphorylation sites, a nuclear localization signal, and an Src homology 3 binding motif (Fig. 1A) (8, 9). Following cytosolic proteolysis of hIL-16 by caspase-3, a 121-amino acid fragment encompassing the C-terminal PDZ domain (residues 527–619) is secreted as the mature form of IL-16 (10). Secreted IL-16 has been reported to bind to CD4 with relatively high affinity (6, 11, 12), which is consistent with IL-16 functioning as a pro-inflammatory cytokine. The protein is reported to have two major effects on CD4+ cells: chemoattraction, preferentially of T1 cells, and inhibition of CD3/T-cell mediated activation, preferentially of T1 cells (13). Co-incubation of CD4+ cells with an anti-CD4 antibody (OKT4) is reported to lead to a reduction in the magnitude of IL-16-induced cell migration by monocytes (11). The protein CD4 contains four immunoglobulin (Ig)-like domains (D1–4), and CD4-derived peptide inhibition studies of IL-16-mediated chemotaxis suggested that IL-16 binds to CD4 D4 (6). There is also evidence to suggest that chemokine receptor 5, which is expressed on the surface of T1 cells, enhances the binding of IL-16 to the co-receptor (14).

Interestingly, the chemotactic activity of hIL-16 is not associated with a characteristic chemokine structural motif (15, 16). The solution structure of mature hIL-16 has been reported and showed the chemokine to contain a classical PDZ domain, consisting of a central up and down β-sandwich, adjacent to an α-helix (17). PDZ domains typically assist in the assembly of multiprotein signaling complexes, by binding peptides in a groove between the α1-helix and β2-strand, in a process known...
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A.

B.

FIGURE 1. IL-16 domain structure. A, IL-16 is expressed as a 631-amino acid precursor protein comprising an N-terminal CcN motif and three C-terminal PDZ domains. Caspase-3 cleaves the precursor protein at Asp^{510}, between PDZ domains 2 and 3, releasing the mature secreted IL-16 component (2). B, multiple sequence alignment of secreted mammalian IL-16 proteins, revealing high sequence homology in the β2 to β3 strand and α1-helix regions. The secondary structure based on human IL-16 is shown above. With the exception of the β3/β4 loop region, the core of the protein has very high sequence homology across all mammalian species. Putative CD4 binding site residues highlighted (*). The figure was prepared using ESPript 3.0 (46).

IL-16 is associated with disease pathogenesis in a number of autoimmune conditions, with expression levels raised in serum from patients with rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus (15, 20, 21). The incubation of dendritic cells with a mouse anti-IL-16 monoclonal antibody (mAb 14.1) was reported to result in a significant reduction in cell migration in cultures of epidermal cells (22). Furthermore, neutralization of hIL-16 by mAb 14.1 produced a reduction in the TH1-type inflammatory response (2) and the mAb 14.1 antibody has shown in vivo efficacy in a rodent model of acute kidney injury (23). The potential of hIL-16 as a therapeutic target in a range of autoimmune conditions has led to the development of a panel of inhibitory monoclonal antibodies. In this communication we report the structure of a promising Fab candidate (c14.1) based on mAb 14.1 bound to hIL-16, which reveals an unexpected binding site for the inhibitory antibody on the opposite face of the protein to the proposed CD4 binding site. In addition, the structure of the c14.1Fab-IL-16 complex reveals a significant conformational change in hIL-16, allowing residues from the CDR loops to make extensive contacts in the generic PDZ domain peptide-binding groove. This raises the possibility of the IL-16 PDZ domain modifying its activity via a cryptic peptide binding site. The structural changes induced in hIL-16 by the inhibitory antibody binding also reveal new opportunities for the design of small molecule inhibitors to target hIL-16 activity.

Experimental Procedures

Expression and Purification of hIL-16—A recombinant protein corresponding to mature secreted human IL-16 (residues 502-631) with an N-terminal His$_{6}$ tag was expressed as a soluble product using a pLEICS-01 vector (Protex, University of Leicester) transformed into the Escherichia coli strain BL21 (DE3) (Novagen). Uniformly $^{15}$C/$^{15}$N/$^{2}$H- and $^{15}$N-labeled hIL-16 were prepared by growing at 37 °C in modified Spizizen minimal medium (24, 25) containing $^{15}$NH$_{4}$SO$_{4}$ (4 g liter$^{-1}$) and/or $[^{13}$C$_{6}$]glucose (2 g liter$^{-1}$) as required, with deuterated samples grown in media dissolved in 100% D$_{2}$O. The protein was purified to homogeneity by chromatography on affinity (nickel-nitrilotriacetic acid column, Qiagen) and gel filtration.
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Expression and Purification of Truncated hIL-16 (hIL-16tr)—The DNA construct for the truncated form of human IL-16 (IL-16tr, residues 523–622) with an N-terminal His tag was cloned into pLEICS-01 and transformed into E. coli BL21 cells. The soluble protein was expressed in 2× YT media at 18 °C and purified to homogeneity by chromatography on affinity (nickel-nitritotriacetic acid column, Qiagen) and gel filtration (Superdex 75 16/60, GE Healthcare) columns, into a final buffer of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 1 mM DTT. The His tag was removed prior to gel filtration using tobacco etch virus protease overnight at 4 °C.

Expression and Purification of the Anti-IL-16 c14.1Fab—The variable domains from the heavy and light chains of the parent murine anti-IL-16 antibody (14.1) were identified by sequence analysis and subcloned into in-house pCMV-based vectors, containing a C-terminal reading frame for either the first constant domain of human IgG1 (C1h1) or the human κ constant domain (Cκ), respectively. For expression of the c14.1Fab, the two plasmids encoding the heavy and light chain fragments were transiently co-transfected into Expi293F™ cells (Invitrogen) according to the manufacturer’s instructions. Cell cultures were maintained for 7–10 days in Expi293™ medium at 37 °C (130 rpm and ≤8% CO2 in humidified air) before supernatants were harvested by centrifugation, filter-sterilized (Stericup™), and stored at 4 °C until required. The secreted c14.1Fab was purified by affinity chromatography, with the harvested supernatant loaded onto a 1-ml KappaSelect column (GE Healthcare) pre-equilibrated with phosphate-buffered saline, pH 7.4, (PBS) and the bound antibody was eluted with 0.1 M glycine, pH 2.5–3.0. The final sample was desalted and buffer exchanged into PBS using a PD10 column (GE Healthcare).

Expression and Purification of Extracellular Regions of Human CD4 (hCD4)—DNA inserts comprising hCD4 domains D1–D4 (residues 1–388) and hCD4 D3D4 (residues 202–388) were synthesized and cloned into pCDNA3 (Life Technologies). The hCD4 D3D4 insert also incorporated an additional N-terminal IgG1 leader sequence to facilitate protein secretion. Both expression vectors also included a C-terminal His6 tag to enable affinity-based protein purification. For recombinant hCD4 expression, Expi293F™ cells (Life Technologies) were transiently transfected with plasmid DNA according to the manufacturer’s instructions. Cell cultures were maintained for 7–10 days in Expi293™ medium at 37 °C (130 rpm and ≤8% CO2 in humidified air) before supernatants were harvested by centrifugation, filter-sterilized (Stericup™; Merck Millipore), and stored at 4 °C until required. The secreted hCD4 was purified by affinity chromatography, with the harvested supernatant loaded onto a 1-ml KappaSelect column (GE Healthcare) pre-equilibrated with phosphate-buffered saline, pH 7.4, (PBS) and the bound antibody was eluted with 0.1 M glycine, pH 2.5–3.0. The final sample was desalted and buffer exchanged into PBS using a PD10 column (GE Healthcare).

Complex Preparation—The c14.1Fab-hIL-16 and c14.1Fab-hIL-16tr complexes were prepared by mixing the c14.1Fab with either hIL-16 or hIL-16tr at a Fab:hIL-16 molar ratio of 1.2:1. The mixtures were incubated for 60 min at 4 °C, concentrated with Vivaspin-20 to a final volume of 2.0 ml, and purified on a Superdex 75 16/60 column (GE Healthcare). The running buffer used was either 25 mM phosphate, pH 7.0, 100 mM NaCl, 100 μM EDTA, 1 mM DTT, and 1 mM imidazole or 20 mM Tris-HCl, pH 7.4, and 50 mM NaCl, for the c14.1Fab-hIL-16 and c14.1Fab-hIL-16tr complexes, respectively. Fractions corresponding to the protein complex peak in each case were pooled and concentrated for NMR and crystallographic studies with a Vivaspin-20. As expected, the complex formation in both cases resulted in a significant shift in retention time on a Superdex 75 column compared with that for the free c14.1Fab and hIL-16.

NMR Spectroscopy—All NMR data were acquired on Bruker Avance III 600 and 800 MHz spectrometers equipped with 5-mm HCN cryoprobes. Both the hIL-16 and c14.1Fab-hIL-16 NMR samples were prepared at 150 μM in 25 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl, 100 μM EDTA, 1 mM DTT, and 1 mM imidazole in 90% H2O, 10% D2O. The two-dimensional 15N-1H TROSY spectra of hIL-16 were obtained at 25 and 35 °C, in the absence or presence of c14.1Fab, respectively. Typical acquisition times for the triple resonance experiments were 80 ms in F3 (1H), 22 ms in F2 (15N), and 6 or 25 ms in F1 (13C) as appropriate, with the spectra collected over 13–60 h. All datasets were non-uniform sample to 25% and reconstructed using hmsIST software (26). Backbone amide chemical shifts for free hIL-16 were consistent with those reported previously. However, all backbone assignments were confirmed using three-dimensional HCNO, CBCACONH, and CBCANH experiments, allowing for the full assignment of peaks from the 15N-1H TROSY spectra. All NMR data were processed and analyzed using NMRPipe (27) and SPARKY (University of California, San Francisco, CA) software.

The minimal shift approach (28) was used to identify hIL-16 residues involved in c14.1Fab binding. Backbone amide minimal shift values were obtained from the combined chemical shift change in 15N and 1H for each assigned peak in the 15N/H TROSY spectrum of the free 15N-labeled hIL-16, when compared with all peaks observed in the 15N/1H TROSY spectrum of the 15N-labeled hIL-16 bound to unlabeled c14.1Fab. A histogram of combined minimal shift versus protein sequence revealed residues from hIL-16 with backbone amide signals significantly perturbed by antibody binding.

Protein Crystallography—Initial crystallization trials for the c14.1Fab-hIL-16tr complex were performed at 20 mg/ml using sitting drop vapor diffusion and 96-well block screens (Molecular Dimensions) at 20 °C. Optimal crystals of c14.1Fab-hIL-16tr grew in 16% PEG3350, 0.1 x Bistris propane, pH 6.5, and 0.2 M sodium sulfate. A cryoprotectant consisting of 22% (v/v) ethylene glycol in mother liquor was used. X-ray diffraction data were collected at the Diamond Light Source (Oxford, UK) using beamline I03.

Diffraction data were indexed and integrated in Xds (29) and scaled using SCALA (30). A Matthews coefficient (31) calculated one molecule of c14.1Fab and one molecule of hIL-16tr...
present in the asymmetric unit. A homology model of c14.1Fab was generated using SWISSMODEL (32) with the deposited antibody structures Protein Data Bank codes 4F33 (33) and 1I7Z (34), used to model the heavy and light chains, respectively. The structure of the c14.1Fab-hIL-16tr complex was determined by molecular replacement, using the program PHASER (35), with the hIL-16 NMR structure (PDB code 1I16) and the c14.1Fab homology model used in the search. Model building and structural refinement was carried out with Coot (36), PHENIX (37), and REFMAC 5.0 (38) using restrained refinement and isotropic B-factors. X-ray data collection and refinement statistics are given in Table 1.

**ELISA-type c14.1mAb-IL-16 Binding Assays—**Wells on streptavidin HBC plates (Thermo Scientific) were coated overnight at 4°C with 50 µl of 0.048–25 µg/ml of recombinant hIL-16 or hIL-16 W600A in Dulbecco’s PBS (Thermo Scientific). After blocking with 2% BSA in PBS at room temperature for 1 h, 50 µl of 1 µg/ml of c14.1 antibody in Dulbecco's PBS with 0.1% BSA was added to the wells and incubated for 1 h at room temperature. Following incubation with HRP-conjugated anti-mouse (Fc specific) antibodies (Sigma A2304) (1:5,000 dilution) at room temperature. Following incubation with HRP-conjugated antibodies (Sigma A2304) (1:5,000 dilution) at room temperature, 50 µl of 3,3′-diaminobenzidine (Sigma, DAB) was added and allowed to develop. Bound proteins were quantified after measuring the absorbance at 450–650 nm in a microtiter plate reader (Tecan Safire), and data were analyzed by GraphPad Prism software.

**Flow Cytometry Analysis of OKT4 Antibody and hIL-16 Binding to Cell Surface CD4—**Flow cytometry analysis of both hIL-16 and OKT4 antibody binding to cell surface CD4 was performed in duplicate, with 1 × 10^6 cells per well in FACs buffer (PBS + 2% FBS). The OKT4 antibody binds to the D3 domain of CD4. CD4+ve cells were incubated with hIL-16 (3.3 ng/ml to 33 ng/ml) for 2 h at 4°C, followed by biotinylated OKT4 (eBioscience 13-0048) at 500 ng/ml final concentration and streptavidin Alexa Fluor 488 (Invitrogen, S11223) at 2 µg/ml final concentration for 30 min at 4°C, then analyzed using a Millipore guava easyCyte.

**Results and Discussion**

**Mapping the Binding Site of the c14.1Fab on hIL-16—**Residues from hIL-16 significantly perturbed by the interaction with the c14.1Fab were identified by comparison of 15N/1H TROSY spectra acquired for the free and antibody bound protein (Fig. 2). Comprehensive backbone and side chain assignments have been previously reported for hIL-16 (17), and it proved relatively straightforward to obtain nearly complete backbone resonance assignments for free hIL-16 under our experimental conditions. The observed minimal shifts in backbone amide signals of hIL-16 on complex formation are summarized in the histogram shown in Fig. 2A and mapped onto the structure of free hIL-16 to clearly identify a contiguous antibody interaction surface. c14.1Fab binding to hIL-16 results in very significant shifts (N-H ∆δ ≥ 0.15 ppm) for 15 residues (Gly542, Gly544, Ser546, Gly549, Gly550, Ser553, Asp557, Thr561, Arg564, Met591, Arg596, Trp600, Asn601, Ile603, and Ala605), which form the interaction surface highlighted in Fig. 2B. These residues are predominantly localized to the β2 and β3 strands, the β2/β3 loop, and the neighboring α1-helix, collectively forming a continuous patch of ~683 Å² on the surface of hIL-16, which is consistent with the size expected for an antibody binding site (39, 40). Surprisingly, the antibody interaction surface identified does not include the residues previously reported to be involved in binding to CD4 (Arg616, Arg617, and Lys618), suggesting that the inhibitory activity of c14.1Fab is not due to direct blocking of CD4 binding to hIL-16.

**Structure of the c14.1Fab-hIL-16 Complex—**To further characterize the interaction of the c14.1Fab with hIL-16 and the molecular basis of the inhibition of activity, the structure of the c14.1Fab-hIL-16 complex was solved by x-ray crystallography to 2.1 Å. The published solution structure of mature hIL-16 (PDB 1I16) revealed highly flexible regions on either end of the central PDZ3 domain (17). In addition, the NMR interaction studies reported here show that these regions are not involved in c14.1Fab binding and that the PDZ3 domain alone contained the entire antibody interaction site. Consequently, an isolated PDZ3 domain hIL-16 construct was prepared corresponding to residues 523–622 (hIL-16tr) and assessed for crystallographic studies. Size exclusion chromatography confirmed that this truncated form of hIL-16 (hIL-16tr) still bound with high affinity to the c14.1Fab molecule and crystals of the antibody-hIL-16tr complex were obtained that diffracted to 2.1 Å.

Analysis of the diffraction data indicated that the crystals contained one molecule of IL-16tr-c14.1Fab complex in the asymmetric unit. Interpretable electron density was obtained for hIL-16tr residues Glu527 to Ser619, c14.1Fab heavy chain (VH) residues Glu1 to Ser223, and c14.1Fab light chain (VL) residues Asp1 to Cys218. The c14.1Fab is a chimeric version of the mouse derived 14.1 inhibitory anti-IL-16 monoclonal antibody. The sequences of the three complementarily determining regions (CDR) in the light (L1, L2, L3) and heavy chains (H1, H2, and H3) can be classified as L1 (Arg24-His32), L2 (Tyr53-Ser68), and H3 (Ser99-Tyr110), respectively (Chothia numbering system).
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The structure of the protein complex shows hIL-16tr bound to the conventional antigen binding surface on the c14.1Fab (Fig. 3). The overall structure of the antibody-bound hIL-16tr molecule is very similar to the published hIL-16 free structure (Cα root mean square deviation 2.09 Å), with the exception of residues 569–575, which appear to be disordered in the crystal structure. The most notable difference in the backbone was for Leu554 (1.0 Å in Cα positions), which is situated in the β2/β3 loop that was shown to be dynamic in solution (17). In complex with the c14.1Fab the hIL-16 β2/β3 loop interacts with CDR-H1, which is likely to stabilize this loop region of hIL-16 and may induce a change in conformation compared with the free protein.

Features of the Antibody-IL-16 Interface—The c14.1Fab-IL-16tr interface buries 876 Å² of antibody protein surface from bulk solvent, with 603 Å² of the buried surface from the VH domain and 273 Å² from the VK domain. In the case of IL-16 a comparable surface of 853 Å² is buried by complex formation. This is consistent with the contact surface area estimated from the NMR studies discussed earlier and lies within the typical range of 850 ± 130 Å² for an antibody-binding site on target proteins (39, 40). With the exception of CDR-L3, at least one residue from all CDR loops is within 5 Å of hIL-16, with 22 amino acids from the CDR loops losing at least 10 Å² of solvent accessibility and making direct contacts with hIL-16. The extensive interface features a mixture of polar and hydrophobic contacts, including the involvement of eight aromatic side chains in the CDR loops, from both the VH (Tyr52, Tyr74, Tyr121, Tyr122, and Tyr127) and VK (Tyr59, Tyr61, and Tyr78) domains. Of these, all are forming hydrogen bonds through their side chain hydroxyl groups, with the exception of Tyr59, Tyr61, and Tyr121. Examples of the interactions seen between the antibody and hIL-16 are illustrated in Fig. 4.

There are 25 residues from hIL-16 within 5 Å of c14.1Fab in the complex, which form a continuous contact surface comprising β-strands β2 and β3, the β2/β3 loop, and α1 helix (Fig. 3B). This antibody binding site on hIL-16 contains many of the residues, which show substantial changes in the backbone amide chemical shifts on complex formation (Fig. 2A and 2B), including residues Gly542, Gly544, Ser546, Ser553, Arg564, Arg596, Trp600, and Ile603. However, significant shifts in backbone amide signals were also seen for several residues not directly involved in antibody binding such as residues Gly549, Gly550, Met591, Asn601, and Ala605. This probably reflects the conformational change in hIL-16 induced by antibody binding and discussed below (Fig. 5).

The c14.1Fab interaction surface on hIL-16 includes several basic residues, such as Arg564 and Arg596, which gives the binding site on hIL-16 an overall positive charge. The complimentary face of the antibody includes four acidic residues from the CDR loops (Asp100, Asp103, and Asp109 from CDR-H3; and Glu59 from CDR-L2). Of these, Asp100 and Glu59 form salt bridges with the antibody.
bridges with Arg596 and Arg564, respectively, as illustrated for Asp100 and Arg596 in Fig. 4B, and which contributes to both the specificity and stability of the complex formed. In total there are 10 salt bridges and hydrogen bonds between hIL-16 and c14.1Fab, together with 13 bridging water molecules, which collectively form a complex hydrogen-bonding and ionic interaction lattice between the two proteins.

A major contributor to the specificity and affinity of the recognition of hIL-16 by the c14.1Fab is shape complementarily at the center of the binding site, driven predominantly by residues in CDR-H3 (Tyr101, Tyr102, and Asp103 (Fig. 4)). These CDR residues occupy the GLGF cleft on hIL-16, which is a structural feature typical of PDZ domains that commonly bind C-terminal peptides (42). The previously reported solution structure of free hIL-16 indicated that this cleft on the hIL-16 was too small to accommodate peptide binding or other potential ligands and was also occluded by the indole side chain of Trp600. In addition, there was no evidence of significant mobility of this region in the free hIL-16 solution structures, nor have any peptides been identified that bind to IL-16. Interestingly, the structure of the c14.1Fab:hIL-16 complex reveals a significant antibody-induced conformational change in the GLGF region of hIL-16, which allows this cleft to accommodate residues from CDR-H3. The localized structural changes observed include a 2 Å shift of the β2-strand to widen the binding site, and a 30° rotation of the α1-helix to lever the Trp600 side chain from the blocked recognition groove (Fig. 5). These antibody-induced conformational changes in bound hIL-16 permit the phenolic side chain of Tyr102 from CDR-H3 to occupy the remodeled hIL-16 GLGF cleft, formed by Phe545, Ser546, Leu547, Arg596, Ala599, Trp600, and Ile603. The acidic side chain of Asp103 from CDR-H3 forms a hydrogen bond with the amide backbone of Gly544, in a similar manner to the carboxyl terminus of a typical peptide binding to the GLGF recognition motif of a PDZ domain.
likely is that a more substantial interface formed with a protein partner could include a peptide moiety binding within this site as part of a larger contact surface area. Indeed, a conformational change of a PDZ domain to accommodate an interaction partner has been observed previously in the Crbβ2 peptide binding to the Pals1 PDZ domain (43). In this instance, Phe318 (occupying the same position as Trp600 in hIL-16) sterically blocks the PDZ-binding groove in the ligand-free structure, but adopts a different rotomer conformation away from the groove to accommodate the Crb17 peptide. Furthermore, the phenyl side chain of Phe318 packs against residues Arg1404 and Leu1405 from Crb17, forming one side of the pocket into which the peptide binds.

**Potential Importance of Trp600 in Target Recognition**—To further probe the importance of the Trp600 side chain in regulating the binding of target protein/peptides to the GLGF region of hIL-16 we produced a W600A variant of hIL-16 and determined the effects on c14.1 antibody binding using an ELISA-based assay. The hIL-16 W600A mutation resulted in a substantial change in the affinity of the interaction between the c14.1 antibody and hIL-16. Removing the blocking tryptophan side chain by substituting the residue with alanine might be expected to increase the affinity of the c14.1 antibody to hIL-16. However, the affinity was observed to decrease by ~10-fold, from an EC_{50} of 115 nM for native hIL-16 (supplemental Fig. S1). This suggests that the energetic penalty arising from the conformational change induced in hIL-16 on antibody binding is more offset by the network of Van der Waals interactions between the c14.1Fab CDR-H3 loop residues and the hydrophobic pocket formed by hIL-16 residues Phe545, Leu547, Arg596, Ile603 and, most importantly, the indole side chain from Trp600 (Fig. 4B).

**Impact of the c14.1Fab on IL-16 Signaling**—The location of the CD4 binding site on hIL-16 was previously probed by assessing the inhibition of IL-16-mediated chemotaxis by linear peptides derived from IL-16, which suggested a role for the 616RRKSK619 motif (19). This putative CD4 binding site is located on the opposite face of hIL-16 to the c14.1Fab binding site reported here. This raises intriguing questions regarding the mechanistic basis of the inhibitory activity of the c14.1 antibody.

Minimal shift NMR experiments were used to assess the binding of CD4 domains D1–D4 and CD4 D3D4 to ^15N-labeled hIL-16 and to establish if CD4 binding was affected by c14.1Fab binding. Surprisingly, in samples with a 5:1 molar excess of either CD4 D1–D4 or D3D4 we observed no shifts in the backbone amide signals of hIL-16, indicating no significant interaction between the two proteins under the experimental conditions used (supplemental Fig. S2). In agreement with the previously published NMR structural studies of hIL-16, the line widths of the backbone amide NMR signals observed are consistent with a predominantly monomeric hIL-16. Interestingly, some reports suggest that CD4 only binds to multimeric forms of hIL-16 (44), however, the inhibitory c14.1Fab clearly binds to monomeric hIL-16 with high affinity.

Attempts to directly or indirectly observe hIL-16 binding to cell lines expressing CD4 by flow cytometry also detected no interaction between CD4 and hIL-16 (supplemental Fig. S3). Fluorescence-activated cell sorting (FACS) experiments showed that recombinant hIL-16 was unable to compete off bound anti-CD4 antibody OKT4, despite concentrations of hIL-16 up to 100 mg/ml. Previous studies have suggested that IL-16 dimerization or tetramerization may be a requirement for CD4 binding and chemotactant activity, which could account for the lack of CD4 binding observed here, however, it is unclear as to why mammalian expressed IL-16 would be multimeric. The purified mature hIL-16 expressed in *E. coli* and used for the work reported here shows no tendency to form multimeric species at concentrations over 1 mM, which may suggest that the multimeric forms of IL-16 reported in mammalian cell extracts may involve other protein partners. However, the inhibitory c14.1Fab clearly binds with high affinity to the *E. coli* expressed hIL-16 monomer and neither sterically blocks or perturbs the proposed CD4-binding site on hIL-16.

**Structure-based Design of Small Molecules to Target the IL-16 Functional Site Recognized by c14.1Fab**—The innovative use of antibodies and antibody fragments to identify opportunities for small molecule drug development on a range of proteins selected as attractive therapeutic targets has been proposed recently (45). This includes the identification of antibodies that modulate target protein activity by binding to regulatory sites, with structures obtained for these antibody-target protein complexes used to inform and guide the development of small molecule inhibitors or activators as potential drugs. The structural studies of the c14.1Fab-hIL-16 complex reported here represent an excellent example of the potential of antibody-assisted approaches, with the conformational changes induced by antibody binding revealing new opportunities for small molecule modulation of IL-16 activity. This method of probing the surface of target proteins, using antibodies as tools, to find allosteric and functional pockets is an innovative and exciting prospect and has the potential to revolutionize drug discovery for a wide range of protein targets.
Author Contributions—G. H., A. M., C. K., R. B., and M. C. coordinated the study. G. H. and M. C. wrote the paper. G. H. and R. C. purified IL-16 protein, E. C., J. A., and K. S. purified c14.1 Fab and CD4 proteins, and G. H. determined the c14.1 Fab:IL-16tr complex X-ray structure. G. H. and F. M. designed, performed, and analyzed the NMR experiments shown in Fig. 2 and supplemental Fig. S2. J. A. designed and performed the ELISA shown in supplemental Fig. S1. W. C. provided the c14.1 Fab DNA construct. S. F. and D. T. designed, performed, and analyzed the FACS experiments shown in supplemental Fig. S3. All authors reviewed the results and approved the final version of the manuscript.

References


SUPPLEMENTARY FIGURE 1. c14.1 antibody binding to plate-bound hIL-16 constructs using an ELISA-type binding assay. Microtiter wells were coated with either hIL-16 (closed blue circles) or hIL-16 W600A (closed green squares) at various concentrations to determine their binding specificity for c14.1 antibody. The c14.1 antibody was detected using a secondary anti-Fc antibody conjugated to HRP (1:5000) and then developed. Data were analyzed by GraphPad Prism software. The ELISA binding study showed that mutating residue Trp600 to alanine in hIL-16 resulted in a ~10-fold increase in the EC50 value from 115.1 nM to >858.7 nM, demonstrating the importance of Trp600 in the interaction interface.

SUPPLEMENTARY FIGURE 2. Absence of a measurable direct interaction between hIL-16 and CD4 by NMR. Shown are overlaid 15N-1H HSQC spectra of 15N-labelled hIL-16 both in the absence (blue) and presence (red) of recombinant unlabelled hCD4 domains D1-D4. The spectra overlay very closely, showing no discernable shifts in the spectra of 15N-labelled hIL-16 in the presence of 5-molar excess of CD4, suggesting no interaction between hIL-16 and CD4.

SUPPLEMENTARY FIGURE 3. Lack of detectable binding of IL-16 to cell surface CD4. (A) CD4 +ve Jurkat cells were stained with a dose response curve of Biotinylated IL-16 (33mg/mL, 3.3mg/mL, 300ng/mL, 33ng/mL, 3.3ng/mL) or Biotinylated OKT4 antibody, (500ng/mL) and subsequently detected with Streptavidin AlexaFluor 488. Red histograms OKT4 antibody staining, blue histograms biotinylated IL-16, green Streptavidin AlexaFluor 488 only. (B) Displacement of OKT4 from CD4+ve SupT1 cells. Red histograms OKT4 antibody staining, (500ng/mL), blue histograms hIL-16 dose response curve at 100mg/mL, 25mg/mL, 10mg/mL, 2.5mg/mL, 1mg/mL, green Streptavidin AlexaFluor 488 only. The FACS data indicate no direct binding of hIL-16 to CD4.
SUPPLEMENTARY FIGURE 1.

<table>
<thead>
<tr>
<th>Sigmoidal dose-response (variable slope)</th>
<th>hIL-16 wild-type</th>
<th>hIL-16 W600A</th>
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<tr>
<td><strong>Best-fit values</strong></td>
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<tr>
<td>EC50 (nM)</td>
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<td>858.7</td>
</tr>
</tbody>
</table>

![Graph showing sigmoidal dose-response curves for hIL-16 wild-type and hIL-16 W600A](image)
SUPPLEMENTARY FIGURE 3.

A.

B.
Structure of a Potential Therapeutic Antibody Bound to Interleukin-16 (IL-16): MECHANISTIC INSIGHTS AND NEW THERAPEUTIC OPPORTUNITIES
Gareth Hall, Eilish Cullen, Kovilen Sawmynaden, Joanne Arnold, Simon Fox, Richard Cowan, Frederick W. Muskett, David Matthews, Andrew Merritt, Catherine Kettleborough, William Cruikshank, Debra Taylor, Richard Bayliss and Mark D. Carr

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