**Conformational Dynamics in Interleukin 17A and 17F Functional Complexes is a Key Determinant of Receptor A Affinity and Specificity**

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**Abstract**

The proinflammatory cytokines IL-17A and IL-17F have been identified as key drivers of a range of human inflammatory diseases, such as psoriasis, which has led to several therapeutic antibodies targeted at IL-17A. The two cytokines have been shown to tightly associate as functional homo and hetero dimers, which induce signalling via the formation of a cell surface signalling complex with a single copy of both IL-17RA and IL-17RC. Striking differences in affinity have been observed for IL-17RA binding to IL-17AA, IL-17AF and IL-17FF, however, the functional significance and molecular basis for this has remained unclear. We have obtained comprehensive backbone NMR assignments for full length IL-17AA (79%), IL-17AF (93%) and IL-17FF (89%), which show that the dimers adopt almost identical backbone topologies in solution to those observed in reported crystal structures. Analysis of the line widths and intensities of assigned backbone amide NMR signals has revealed striking differences in the conformational plasticity and dynamics of IL-17AA compared to both IL-17AF and IL-17FF. Our NMR data indicate that a number of regions of IL-17AA are interconverting between at least two distinct conformations on a relatively slow timescale. Such conformational heterogeneity has previously been shown to play an important role in the formation of many high affinity protein-protein complexes. The locations of the affected IL-17AA residues essentially coincides with the regions of both IL-17A and IL-17F previously shown to undergo significant structural changes on binding to IL-17RA. Substantially less conformational exchange was revealed by the NMR data for IL-17FF and IL-17AF. We propose that the markedly different conformational dynamic properties of the distinct functional IL-17 dimers plays a key role in determining their affinities for IL-17RA, with the more dynamic and plastic nature of IL-17AA contributing to the significantly tighter affinity observed for binding to IL-17RA. In contrast, the dynamic properties are expected to have little influence on the affinity of IL-17 dimers for IL-17RC, which has recently been shown to induce only small structural changes in IL-17FF upon binding.

Key words: Interleukin-17, conformational dynamics, receptor affinity, structural changes, Interleukin-17 receptor

Abbreviations: IL-17RA, Interleukin-17 Receptor A; IL-17C, Interleukin-17 Receptor C; D2O, Deuterium Oxide; DTT, Dithiothreitol; NP40, Nonidet P-40; DNase I, Deoxyribonuclease 1; MWCO, Molecular Weight Cut Off; UPLC, Ultra Performance Liquid Chromatography; TCEP, Tris(2-carboxyethyl)phosphine; QTof, Quadrupole Time-of-Flight; NiNTA, Nickel Nitrilotriacetic Acid; HSQC, Heteronuclear Single Quantum Coherence, NOESY, Nuclear Overhauser Effect Spectroscopy; TROSY, Transverse Relaxation Optimized Spectroscopy; NOE, Nuclear Overhauser Effect; IST, Iterative Shrinkge Thresholding; HDX-MS, Hydrogen-Deuterium Exchange – Mass Spectrometry; SPR, Surface Plasmon Resonance

1. **Introduction**

The success of secukinumab and ixekizumab in treating psoriasis, ankylosing spondylitis and psoriatic arthritis has highlighted the importance of IL-17 biology in autoimmune inflammatory disease, with IL-17A now well established as a clinically validated target (Griffiths *et al.* 2015, Thaci *et al.* 2015). The family of IL-17 cytokines also includes IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F, all of which share some homology in primary sequence, most striking between IL-17A and IL-17F (55% sequence homology) (Hymowitz *et al.* 2001). In particular, four cysteine residues and two serine residues are completely conserved across family members, providing the hallmark cysteine knot fold tertiary structure. Structural studies with IL-17A and IL-17F have shown that they exist both as homo- (IL-17AA and IL-17FF) and hetero-dimers (IL-17AF), with interactions with the common receptor IL-17RA occurring over a large polar interface (Hymowitz et al. 2001, Chang *et al.* 2007, Ely *et al.* 2009, Liu *et al.* 2013, Goepfert *et al.* 2017). A second receptor chain, IL-17RC in the case of IL-17A and F, provides specificity for subsequent signalling (Gaffen 2009).

Through signalling via a ternary complex formed with IL-17RA and IL-17RC, IL-17A and IL-17F protect against bacterial and fungal infection, especially at mucosal and epithelial surfaces such as the skin. Effector molecules which stimulate barrier repair are upregulated and induce a pro-inflammatory environment, allowing neutrophil accumulation as part of the response to pathogen invasion. However, this inflammation if not well regulated, can lead to inappropriate cutaneous inflammation and tissue damage, with IL-17A and IL-17F also associated with a range of autoimmune conditions (Schett *et al.* 2013). Next generation biological therapies, exemplified by Bimekizumab, which target both IL-17A and IL-17F containing complexes hold further promise for patients suffering from IL-17-dependent autoimmune disease (Glatt *et al.* 2018, Adams 2020).

Comprehensive biophysical studies looking at the interaction of both wild-type and mutant IL-17 dimers with their receptors have shown that significantly different affinities are observed for the binding of IL-17AA, IL-17AF and IL-17FF to IL-17RA and IL-17RC (Wright *et al.* 2008, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017, Goepfert *et al.* 2020). Despite the availability of crystal structures for all of these IL-17 dimers, both in isolation and in complex with IL-17RA, as well as for the IL-17FF/IL-17RC complex, the observed differences in affinities have yet to be fully explained (Hymowitz et al. 2001, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017, Goepfert et al. 2020).

The objective of the study reported here was to characterise the structural features and dynamic properties of functional IL-17A and IL-17F dimers in solution and determine whether potential differences could play a key role in receptor affinity and/or specificity. We obtained comprehensive backbone NMR chemical shift assignments for IL-17AA, IL-17FF and IL-17AF, which reveal that in solution the dimers adopt very similar backbone topologies to those observed in the published crystal structures. Interestingly, a wide range of peak widths and intensities were observed in the NMR spectra of IL-17AA, which suggest that large regions of the homodimer exist in at least two distinct conformational states in equilibrium. Equivalent analysis suggested that significantly smaller regions of IL-17AF and IL-17FF are present in multiple conformations. A number of significant structural changes have previously been shown to occur in IL-17 dimers upon binding of IL-17RA, in particular for IL-17A (Hymowitz et al. 2001, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017). It seems highly likely that the differing dynamic properties of IL-17AA, IL-17AF and IL-17FF play a key role in determining the very distinct affinities of these functional dimers for IL-17RA, with the much greater conformational heterogeneity seen for IL-17AA contributing to the significantly tighter affinity observed for its interaction with IL-17RA.

1. **Material and Methods**

*2.1 Protein Expression and Purification*

Uniformly 15N, 15N/13C, 15N/2H and 15N/13C/2H labelled samples of mature human IL-17AA and IL-17FF homodimers (residues 24-155 and 31-163 respectively) were prepared from a pET32 vector, which introduced a methionine residue at the N-terminus. The vectors were transformed into BL21-DE3 PLysS cells, which were grown in modified Spizizen’s minimal media containing 1 g/l 15N ammonium sulphate and if required 4 g/l 13C glucose as the sole sources of nitrogen and carbon (Karunairatnam *et al.* 1958). Deuterated samples were grown in media prepared using >99% D2O.

Transformants were grown at 37 °C, and expression of the proteins was induced in mid-log phase (corresponding to an absorbance at 600 nm of 0.8) by the addition of 1 mM IPTG. The cells were harvested at 4 hours post induction by centrifugation at 4,000 g for 20 minutes at 4 °C. Cell pellets containing the insoluble IL-17 proteins were resuspended in lysis buffer containing 34 mM Tris-hydrochloride, 500 mM lithium chloride, 40 mM potassium chloride, 20 mM EDTA, 2 mM magnesium chloride, 0.8 mM DTT, 0.4 mg/ml lysozyme, 10% sucrose (v/v) and 0.25% NP40 (v/v) at pH 8.5 to which was added cOmpleteTM Protease Inhibitor tablets (Roche) and a few crystals of bovine DNase I (Sigma). The cells were then lysed using sonication followed by a French Pressure Cell Press (Thermo Fisher Scientific). The insoluble fraction of the cell lysate was recovered by centrifugation at 15,000 g for 30 minutes at 4 °C. The inclusion bodies were washed twice in a 10 mM Tris-hydrochloride, 0.5 M lithium chloride, 0.1 mM EDTA, 1 mM DTT and 0.5% NP40 (v/v) buffer at pH 8.5, followed by a further two times in a 10 mM Tris-hydrochloride, 0.1 mM EDTA, 1 mM DTT and 0.5% NP40 (v/v) buffer at pH 8.5. After the final wash, the IL-17 inclusion bodies were solubilized in a 5 M guanidine-hydrochloride, 50 mM Tris-hydrochloride, 50 mM sodium chloride and 10 mM DTT buffer at pH 8.5, to give a final IL-17 concentration of 2 mg/ml.

The resolubilised IL-17 proteins were refolded by drop-wise, 100-fold dilution into a stirred redox buffer consisting of 50 mM Tris-hydrochloride, 50 mM sodium chloride, 1 mM EDTA, 0.78 mM reduced glutathione, 0.44 mM oxidised glutathione and 0.45 M guanidine-hydrochloride at pH 8.5. After dilution, the refolding mixture was gently stirred for 1 hour at 25 °C followed by a further 16 hours without stirring. Refolded material was filtered and concentrated to a volume of 500 ml using a Vivaflow 200 polyethersulfone (PES) membrane (5000 MWCO; Sartorius) attached to a MasterFlex easy-load pump (Cole Parmer), followed by dialysis into 10 mM MES buffer at pH 6. The refolded IL-17 dimers were loaded onto a 5 ml HiTRAP SP HP cation exchange column (GE Healthcare) preequilibrated with 10 mM MES buffer at pH 6 and the protein eluted by applying a linear gradient between 0 and 1 M sodium chloride. The identity of the IL-17 homodimers and the presence of both the intramolecular (2/protomer) and intermolecular (2/dimer) disulphides were confirmed by mass spectrometry. Reduced and non-reduced samples were run on a Waters Xevo G2 QTof mass spectrometer, which was directly coupled to a Waters Acquity UPLC system under denaturing conditions. Samples of IL-17 dimer were diluted to 1 mg/ml with phosphate buffered saline (PBS) at pH 7.4 and a 15 μl aliquot was then incubated for 40 minutes with 45 μl of either 6.65 mM TCEP, 200 mM ammonium acetate buffer at pH 7.4 (reduced sample) or PBS at pH 7.4 (non-reduced). 5 μl samples were injected onto a Waters BioResolve RP mAB Polyphenyl 450 Å, 2.7 µm, 2.1 x 150 mm column, which had been preequilibrated in 95% aqueous mobile phase (0.02% trifluoracetic acid, 0.08% formic acid in water) and 5% organic mobile phase (95% acetonitrile, 5% water, 0.02% trifluoracetic acid, 0.08% formic acid). The IL-17 dimers were eluted on a linear gradient from 95% aqueous mobile phase:5% organic mobile phase to 5% aqueous mobile phase:95% organic mobile phase.

To facilitate the assignment of the IL-17A/IL-17F heterodimer, NMR samples of the dimer where only IL-17A or IL-17F was 15N/13C/2H labelled were prepared. These samples were produced by mixing resolubilized His-tagged IL-17A and IL-17F together prior to refolding. To aid the purification of the IL-17AF heterodimer, the IL-17A protomer was cloned into an in-house pET-based vector, which introduced a N-terminal hexa-His-tag and TEV-cleavage site to the construct. The untagged IL-17F construct that was used to prepare samples of the homodimer was also used for the IL-17AF heterodimer.

The His-IL-17A and IL-17F monomers were expressed, isolated and resolubilized separately as described for the IL-17 homodimers. The resolubilized His-IL-17A and IL-17F were mixed together at a 1:2 ratio to give a final concentration of 2 mg/ml and then refolded as described for the homodimers. In order to separate the His-IL-17AF heterodimer from any refolded homodimers, the proteins were dialysed into 20 mM Tris, 200 mM sodium chloride and 20 mM imidazole buffer at pH 7.5, filtered and loaded onto a 5 ml NiNTA column (Superflow cartridge; Qiagen), which had been preequilibrated in the same buffer. The untagged-IL-17FF homodimer passed straight through the column without binding, whilst the His-tagged dimers were eluted by the application of a linear gradient between 20 and 500 mM imidazole. Due to the His-IL-17A:IL-17F ratio present at the refolding step, only a small amount (<10%) of His-IL17AA homodimer was formed. The presence of two His-tags on the His-IL-17AA homodimer resulted in the His-IL-17AA eluting at a higher imidazole concentration than was seen for the single His-tagged IL-17AF heterodimer. As a result, SDS-PAGE analysis of the elution fractions allowed the His-IL-17AF heterodimer to be successfully separated from the His-IL-17AA homodimer. The identity of the His-IL-17AF heterodimer and the presence of both the intramolecular (2/protomer) and intermolecular (2/dimer) disulphides was confirmed by comparison of reduced and non-reduced samples by SDS-PAGE and mass spectrometry.

*2.2 Sequence Specific Assignment of the Backbone NMR Signals of IL-17AA, IL-17FF and IL-17AF*

NMR spectra of the IL-17 homodimers were acquired from 0.35 ml samples of 300-450 μM IL-17AA and 90-210 μM IL-17FF in a 20 mM sodium phosphate, 100 mM sodium chloride and 0.02% (w/v) sodium azide buffer at pH 6.0, containing 5% D2O/95% H2O. All NMR data were acquired at 35 °C on either a 600 MHz Bruker Avance/DRX or 800 MHz Bruker Avance II/AVII spectrometer. The 2D and 3D spectra recorded to obtain sequence specific assignments for IL-17AA and IL-17FF were: 15N/1H HSQC (Bodenhausen *et al.* 1980); NOESY-HSQC (Marion *et al.* 1989) with an NOE mixing time of either 600 ms (IL-17AA) or 200 ms (IL-17FF); 15N/13C/1H TROSY-HNCACB (Wittekind *et al.* 1993, Salzmann *et al.* 1999), TROSY-HN(CO)CACB (Salzmann et al. 1999), TROSY-HNCA (Grzesiek *et al.* 1992, Salzmann et al. 1999), TROSY-HN(CO)CA (Salzmann et al. 1999) and TROSY-HNCO (Grzesiek et al. 1992, Salzmann et al. 1999). Typical acquisition times in F1 and F2 for the 3D experiments were 12-22 ms for 15N, 6-8 ms for 13C (25 ms for HNCO experiments) and 22 ms for 1H, with an acquisition time of 35-60 ms in F3 (1H). Typical acquisition times in 2D experiments were 46-68 ms for 15N (F1) and 47-60 ms for 1H (F2).

NMR spectra of the His-IL-17AF heterodimer were acquired from 0.36 ml samples of 260 μM 15N/13C/2H labelled His-IL-17A in complex with unlabelled IL-17F (IL-17Af) and 110-400 μM unlabelled His-IL-17A in complex with 15N/13C/2H labelled IL-17F (IL-17aF) in a 20 mM sodium phosphate, 100 mM sodium chloride and 0.02% (w/v) sodium azide buffer at pH 6.0, containing 5% D2O/95% H2O. All NMR data were acquired at 35 °C on either 600 MHz Bruker AVIII or 800 MHz Bruker AVII spectrometers. The 2D and 3D spectra recorded to obtain sequence specific assignments for His-IL-17AF were: 15N/1H TROSY (Pervushin *et al.* 1998); NOESY-HSQC (IL-17aF) (Marion et al. 1989) or NOESY-TROSY (IL-17Af) (Marion et al. 1989, Pervushin et al. 1998) with an NOE mixing time of 200 ms; 15N/13C/1H TROSY-HNCACB (Wittekind et al. 1993, Salzmann et al. 1999), TROSY-HN(CO)CACB (Salzmann et al. 1999) and TROSY-HNCO (Grzesiek et al. 1992, Salzmann et al. 1999). Typical acquisition times in F1 and F2 for the 3D experiments were 14-22 ms for 15N, 7 ms for 13C (25-30 ms for HNCO experiments) and 17 ms for 1H, with an acquisition time of 60 ms in F3 (1H). Typical acquisition times in 2D experiments were 50 ms for 15N (F1) and 80 ms 1H (F2). The 15N/1H NOESY-HSQC and 15N/13C/1H triple resonance experiments (except the TROSY-HNCO for IL-17Af) were acquired using non-uniform sampling, with the datasets sparsed at 40-44% and 27% respectively.

The majority of the 3D spectra were collected over approximately 64-90 hours. The WATERGATE method was used to suppress the water signal when required. The 3D NMR data were processed using either Topspin (Bruker Biospin Ltd) or NMRPipe (Delaglio *et al.* 1995) with linear prediction used to extend the effective acquisition times by up to 2-fold in nitrogen.  The non-uniform sampled data was reconstructed using the IST algoritm within NMRPipe (Delaglio). For IL-17AA and IL-17FF, HSQC spectra were referenced to water at 35 °C, with TROSY-based triple resonance spectra then referenced to the HSQC spectra. 2D TROSY and 3D TROSY-based triple resonance spectra for the His-IL-17AF heterodimer were referenced to the C-terminal residue of the appropriate homodimer. In the carbon dimension no correction factors were applied to compensate for the deuterium effect. The spectra were analysed using the Sparky package (Lee *et al.* 2015, Goddard *et al.* unpublished).

Sequence-specific backbone resonance assignments (N, NH, C Cand CO) were obtained for IL-17AA, IL-17FF and His-IL17AF from the identification of intra and inter-residue connectivities in TROSY-HNCACB, TROSY-HN(CO)CACB, TROSY-HNCA, TROSY-HN(CO)CA and TROSY-HNCO spectra. To confirm the assignments and resolve any ambiguities, 15N/1H NOESY-HSQC or NOESY-TROSY spectra with longer than normal mixing times were acquired on 15N/2H-labelled IL-17AA, 15N/2H-labelled IL-17FF, 15N/13C/2H-labelled His-IL-17A/unlabelled IL-17F and unlabelled His-IL-17A/15N/13C/2H-labelled IL-17F in order to detect long range amide proton-amide proton NOEs present between sequential residues, and across sheets and loops.

*2.3 Measurement of the Relative Intensities of Backbone Amide Signals of IL-17AA, IL-17FF and IL-17AF*

15N/1H HSQC spectra (Bodenhausen et al. 1980) of the IL-17AA, IL-17FF, IL-17Af and IL17aF dimers were acquired from 0.35 ml samples of 62-75 μM 15N/13C/2H or 15N/2H-labelled dimer in a 20 mM sodium phosphate, 100 mM sodium chloride and 0.02% (w/v) sodium azide buffer at pH 6.0, containing 5% D2O/95% H2O. Spectra were acquired at 35 °C on a 600 MHz Bruker AVIII HD spectrometer. Acquisition times were 60 ms for 15N (F1) and 80 ms 1H (F2), with the spectra acquired over approximately 11 hours. The spectra were processed using NMRPipe (Delaglio et al. 1995) and the height of the backbone amide signals determined in NMRFAM-Sparky (Lee et al. 2015). Significantly overlapped signals, whose intensity could not accurately be determined, were excluded from subsequent analysis. To correct for differences in the concentrations of the NMR samples, the backbone amide peak heights obtained for IL-17FF, IL17Af and IL17aF were scaled to the data obtained for IL-17AA based on the intensity of the signal from the C-terminal residue. The mean signal intensity (1.13x107) and standard deviation (4.67x106) of residues in the four main ß-strands of all three dimers were calculated. Residues with a backbone amide signal intensity of less than one standard deviation below the mean (< 6.62x106) were considered to be significantly weaker than expected, which is indicative of conformational exchange (Cavanagh *et al.* 2007, Marintchev *et al.* 2007).

1. **Results**

*3.1 Assignment of the Backbone NMR Signals of IL-17AA, IL-17AF and IL-17FF*

A wide range of peak widths and intensities were observed in NMR spectra of the IL-17AA homodimer, with a significant number of expected NMR signals being either unobservable or significantly broadened, suggesting that they come from regions of IL-17AA that exist in at least two distinct conformational states that interconvert on a relatively slow timescale (Supplementary Figure 1A) (Cavanagh et al. 2007, Marintchev et al. 2007). Despite the range of linewidths observed, we were able to confidently assign the majority of the protein backbone of IL-17AA (78% of backbone amide signals, 80% of Cα and Cβ and 78% of CO signals). The majority of the residues whose NMR signals were either missing, or we were unable to confidently assign, were located in two regions: β-strand 0 (β0) and the following flexible coil region (residues F41-T56), and the turn between β-strands β3-β4 and adjacent residues (residues R124-R134) (Figure 1A).

Timeline

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**Figure 1. Locations of Secondary Structure Elements in IL-17AA, IL-17FF and IL-17AF.** Panel A shows the amino acid sequence of IL-17A. Indicated above the sequence are the regions of IL-17AA predicted to be extended (purple) or helical (pink) by analysis of the backbone NMR assignments obtained using TALOS-N (Shen *et al.* 2013). Regions observed to be extended (blue) or helical (red) in the crystal structure of IL-17AA (PDB code 4HR9) are shown above for comparison. Regions for which no NMR assignments could be obtained, or were not visible in the crystal structure data, are shown as dotted lines. Equivalent NMR and X-ray based results for the IL-17A subunit of IL-17AF (PDB code 5N92) are shown below the IL-17A sequence. Panel B shows the amino acid sequence of IL-17F. The regions of regular secondary structure predicted by TALOS-N analysis of the backbone NMR assignments obtained for IL-17F in both IL-17FF and IL-17AF, together with elements observed in the crystal structures of IL-17FF (PDB code 1JPY) and IL-17AF (PDB code 5N92), are indicated above and below the IL-17F sequence respectively, as in panel A.

In contrast to the IL-17AA homodimer, the IL-17A protomer of the IL-17AF heterodimer (referred to as IL-17Af) gives rise to relatively well-resolved spectra, with more uniform linewidths and intensities, as illustrated in the 15N/1H HSQC spectrum shown in Supplementary Figure 1B. Essentially complete backbone resonance assignments were obtained for IL-17Af (92% of backbone amide signals, 90% of Cα and Cβ and 87% of CO signals). Whilst significantly fewer broad signals or unassigned residues were observed in IL-17Af, the areas affected were again located in the long flexible coil region and the β3-β4 turn (residues H52-N57 and H128-N131) (Figure 1A).

IL-17FF and the IL-17F protomer of the IL-17AF heterodimer (IL-17aF) also give rise to relatively well-resolved spectra, as illustrated by the 15N/1H HSQC spectra shown in Supplementary Figures 1C and 1D. This allowed essentially complete backbone resonance assignments to be made for IL-17FF (88% of backbone amide signals, 90% of Cα and Cβ and 85% of CO signals) and His-IL17aF (95% of backbone amide signals, 98% of Cα and Cβ and 91% of CO signals). In both cases, the very small number of unassigned backbone amides are located in either the N-terminal region or the β3-β4 turn (Figure 1B).

The comprehensive 15N, 13C and 1H resonance assignments obtained for IL-17AA, IL-17FF and His-IL-17AF have been deposited at the BioMagResBank database (accession numbers 50537, 50538 and 50540 respectively).

*3.2 Comparison of the Crystal and Solution Structures of IL-17AA, IL-17AF and IL-17FF*

The crystal structures of IL-17AA, IL-17FF and IL-17AF have previously been determined, revealing that each protomer of these dimeric cytokines displays a cysteine-knot-like fold, primarily consisting of two β-hairpins (Figure 2C and Supplementary Figure 2) (Hymowitz et al. 2001, Liu et al. 2013, Goepfert et al. 2017). Strands β3 and β4 which make up the second of these β-hairpins form a major part of the dimer interface. The structure of the IL-17 dimers has previously been likened to a garment, with the second β-hairpin being described as the ‘body’(Hymowitz et al. 2001). Strands β1 and β2 form the exterior, first β-hairpin, and are referred to as the ‘sleeves’ of the garment. The two hairpins are joined in the ‘collar’ region by two intramolecular disulphides, although the third disulphide that is required to finish the knot fold is absent. The predominantly unstructured N-terminal region, together with a small part of the second β-hairpin, form the ‘skirt’ of the garment. This region contains an intermolecular disulphide, as well as an additional β-strand (β0), which also forms part of the dimer interface. The N-terminal region is connected to the structured core of the protomer via a long, poorly conserved, coil region that crosses the dimer interface (Supplementary Figure 2A).

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**Figure 2. Analysis of Backbone Amide NMR Assignments Obtained for IL-17AA, IL-17FF and IL-17AF.** Panel A shows an overlay of 15N/1H TROSY spectra acquired from 15N/13C-labelled IL-17AA (black) and 15N-labelled IL-17A in complex with IL-17F (red). Panel B contains a histogram of the chemical shift differences observed for backbone amide groups of IL-17A in IL-17AA compared to IL-17AF. Combined 15N and 1H chemical shift differences for backbone amides were calculated as described previously (Waters *et al.* 2007). The positions of the 5 main β-sheets in IL-17A are highlighted on the histogram. Equivalent spectra and NMR assignment comparisons for IL-17F in IL-17FF and IL-17AF are shown in panels D) and E). Panel C shows a ribbon representation of IL-17AF (PDB code 5N92), in which residues are coloured according to the backbone amide chemical shift differences seen between equivalent subunits in the heterodimer compared to IL-17AA and IL-17FF. Residues that showed a chemical shift change of less than 0.1 ppm are shown in white, over 0.5 ppm in red and those between on a linear gradient between white and red. Residues for which no chemical shift data were obtained are shown in yellow. Panel F shows the structure of IL-17AF in the same orientation as the left-hand side of panel C. The heterodimer is coloured on the basis of sequence conservation between IL-17A and IL-17F, with identical residues in IL-17A and IL-17F shown in grey and white respectively. Similar residues in IL-17A and IL-17F are coloured in yellow and cyan respectively and non-conserved residues in green and blue. Similar residues were grouped as follows: AVLIM, FYW, KRH, DE, ST, NQ, C, P, G. The locations of the β-strands β0-β4, and the N- and C-termini are labelled.

Analysis of the backbone NMR assignments obtained for IL-17AAusing TALOS-N (Shen et al. 2013) shows that each subunit of the complex in solution contains 4 long regions of extended secondary structure (> 3 residues) (residues P73-E80, V88-K93, P114-R123 and R134-T148). These regions are in close agreement with the position of the β-strands β1-β4 observed in the crystal structure of an N-terminally truncated version of IL-17AA (residues 34-155, 4HR9; (Liu et al. 2013)), as shown in Figure 1A. An additional β-strand (β0) was also observed in this crystal structure (residues T44-N48), however, it should be noted that this region of the protein is stabilised by crystal packing. As no backbone assignments could be obtained for this region of the protein, we were unable to determine the secondary structure for this region in solution. Interestingly, crystallographic studies of full-length IL-17AA in complex with a neutralizing antibody fragment (Fab) revealed that the first 34 residues of IL-17AA, which includes both β0 and the long coil region, were not visible in the electron density, suggesting that this region of IL-17AA is present in multiple conformations (Gerhardt *et al.* 2009). In this complex all the crystal lattice interactions are mediated through the Fab molecules. In addition, HDX-MS studies of apo-IL-17AA showed that this region of the dimer underwent rapid deuterium uptake, suggesting that it is disordered and/or dynamic (Gerhardt et al. 2009, Espada *et al.* 2016).

Similarly, analysis of the backbone chemical shifts obtained for the IL-17A protomer of the IL-17AF heterdimer revealed that there were five long regions of extended secondary structure (residues T44-V47, P73-E80, V88-K93, P114-R123 and F133-T148). This is in close agreement with the positions of β0-β4 observed in the crystal structure of IL-17AF (5N92; (Goepfert et al. 2017)), as shown in Figure 1A.

Although very similar regions of regular secondary structure were observed for the IL-17A protomer in both IL-17AA and IL-17AF, significant backbone amide chemical shift differences were observed in some regions, as shown in Figure 2A and 2B. The majority of the differences occur in the N-terminal region and the second β-hairpin (β3 and β4) (Figure 2B). Analysis of the IL-17AA and IL-17AF crystal structures (Figure 2C) shows that most of these chemical shift changes are located at the dimer interface. It is therefore likely that many of the observed backbone amide chemical shift differences are a result of non-conserved dimer interface contacts between the homo- and heterodimer (Figure 2C, 2F and Supplementary Figure 2). However, the additional regions of the IL-17A protomer exhibiting conformational exchange in IL-17AA compared to IL-17AF are also likely to lead to some significant chemical shift changes (discussed in section 3.3).

Analysis of the backbone chemical shifts obtained for IL-17FF shows that the subunits contain 5 long regions of extended secondary structure (residues M55-I61, P81-D89, V97-C102, V121-K133 and S140-T156). These regions are in close agreement with the position of the β-strands β0-β4 observed in the crystal structure of IL-17FF (1JPY; (Hymowitz et al. 2001)), as shown in Figure 1B. Similarly, analysis of the backbone chemical shifts of the IL17F protomer of the IL-17AF heterodimer revealed five long regions of extended secondary structure (residues S54-D58, P81-D89, V97-C102, P122-Q135, S138-T156), which are in close agreement with the position of β0-β4 observed in the crystal structure of IL-17AF (5N92; (Goepfert et al. 2017)) (Figure 1B).

Comparison of the backbone amide chemical shifts obtained for the IL-17F protomers in the IL-17FF homodimer and the IL-17AF heterodimer revealed striking chemical shift changes for about half of the residues (>0.1 ppm), as shown in Figure 2D and 2E. Most notably, large chemical shift differences were observed in the N-terminal region, β0, long coil region and in strands β3 and β4, which correspond to regions of IL-17 that showed significant structural changes between the crystal structures reported for IL-17FF and IL-17AF (Supplementary Figure 2). In addition, many of the residues that show large chemical shift changes are located in regions of IL-17F involved in non-conserved contacts at the dimer interfaces (Figure 2C and 2F) and compliment changes seen in the equivalent regions of IL-17A. As discussed for IL-17A, the differing amounts of conformational exchange observed for IL-17AF compared to IL-17FF will also contribute to the shifts seen in backbone amide signals (discussed in section 3.3).

*3.3 Identification of Regions of Conformational Exchange in IL-17AA, IL-17AF and IL-17FF*

As previously mentioned in section 3.1, a significant number of IL-17AA NMR peaks were either significantly broadened or unobservable in spectra acquired to assign the protein backbone, which is indicative of regions of a protein in conformational exchange on a relatively slow time scale (typical interconversion rates between 2000-200 s-1) (Cavanagh et al. 2007, Marintchev et al. 2007). The extent of peak overlap in 15N/1H-HSQC spectra of IL-17AA precludes accurate determination of the linewidths for many backbone amide signals (Supplementary Figure 1A), however, the inversely related peak intensity could be reliably determined for most residues allowing the identification of regions of IL-17AA that exist in multiple conformations or show high mobility (Figure 3A). In addition, a small number of residues (~17) in IL-17AA gave rise to two separate backbone amide signals, which indicates the presence of two local conformations that interconvert on a slow timescale (interconversion rate typically less than 200 s-1). When the locations of residues identified as being in conformational exchange were mapped on to the structure of IL-17AA, it became apparent that significant portions of the ‘skirt’, ‘sleeves’ and long flexible coil regions of IL17AA were affected (Figure 4A). It should be noted that it was not possible to map a number of the affected residues in the N-terminal region of IL-17AA on the crystal structure, as these residues were either absent from the truncated construct used for the crystallographic studies, or were not visible in the electron density (T26, I27, R29, G32, C33, N35, S36, E37). Interestingly, one of the cysteine residues that form the cysteine knot (C144) showed a significantly reduced backbone amide NMR signal. The chemical shift of the backbone amide proton of C144 is located relatively close to the proton signal arising from water (5.2 and 4.7 ppm respectively). Consequently, it is not clear whether C144 is in conformational exchange, or whether its backbone amide signal is affected by the water suppression technique used in the NMR experiments.

**Chart, histogram

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**Figure 3. Correlation of Structural Changes Induced in IL-17AA, IL-17AF and IL-17FF by Receptor Binding with Dynamic Regions Identified by NMR.** The histogram in panel A shows the relative peak intensity of apo-IL-17AA backbone amide signals from a representative 15N/1H HSQC spectrum. Residues for which no NMR assignments could be obtained are indicated by red lines, whilst prolines and peaks from residues that were too overlapped to obtain reliable intensities are left blank. The backbone structural changes induced in each subunit of IL-17AA upon binding of IL-17RA are displayed as orange and blue lines. The free (PDB code 4HR9) and IL-17RA-bound IL-17AA (PDB code 4HSA) crystal structures were overlaid on the C⍺ atoms of the highly conserved residues that form the cysteine knot region, with C⍺ RMSDs then determined for all equivalent residues using VMD (Humphrey *et al.* 1996). The RMSD trace for the IL-17A subunit that forms the main interface with the D1 domain of IL1-7RA is shown in orange, while the other subunit is shown in blue. The locations of the five main β-strands seen in IL-17AA are highlighted above the histogram. Panels B, C and D show equivalent results obtained for the IL-17A subunit of IL-17AF (PDB codes 5N92 and 5NAN), both chains of IL-17FF (PDB codes 1JPY and 3JVF) and the IL-17F subunit of IL-17AF (PDB codes 5N92 and 5NAN) respectively. Panel E shows the IL-17FF backbone amide peak intensity data previously shown in panel C but compared to the smaller C⍺-RMSD changes induced by binding to IL-17RC (PDB code 1JPY and 6HG4).

Diagram

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**Figure 4. Distinct Patterns of Conformational Heterogeneity Identified in IL-17AA, IL-17FF and IL-17AF.** Panel A shows a ribbon representation of the backbone topology of IL-17AA (PDB code 4HSA) in which residues with significantly lower than average backbone amide peak intensities in 15N/1H HSQC spectra are highlighted in cyan (at least one standard deviation below the mean intensity of peaks from residues in β1-β4). Residues found to give rise to two separate backbone amide peaks are also highlighted in cyan. Amino acids for which no backbone amide assignment could be obtained are shown in blue, whilst prolines and residues with severely overlapped peaks for which reliable intensities could not be determined are shown in yellow. Panels B and C show equivalent highlighted structures for IL-17FF (PDB code 1JPY) and IL-17AF (PDB code 5N92). In panel C the IL-17A subunit is pictured on the left and the IL-17F on the right. Due to the absence of a significant portion of the long interface coil region in the crystal structure of free IL-17AA, the more complete structure obtained in complex with IL-17RA was used in panel A. For IL-17FF and IL-17AF the free crystal structures are shown. The Figure clearly highlights substantial differences in the dynamics of the functional forms of IL-17A and IL-17F.

In contrast to IL-17AA, the backbone amide peak widths and intensities arising from the IL-17FF and IL-17AF dimers were generally more uniform, suggesting that significantly smaller regions of IL-17FF and IL-17AF are in conformational exchange (Figure 3B-D). Similarly, only a very small number of residues in IL-17FF (5 residues) and IL-17AF (2 residues) were identified as being present in 2 distinct forms. Almost all the missing and broad/weak IL-17FF backbone amide signals are located in the ‘skirt’ region, suggesting that only this region of IL-17FF is significantly affected by conformational exchange (Figure 4B). Relatively few broad/weak or missing backbone amide signals were detected in IL-17AF, with residues in the β3-β4 turn and short helix of both protomers, as well as the long flexible coil region of IL-17A bound to IL-17F being the main affected regions (Figure 4C). In contrast to both homodimers, very little conformational exchange was detected in the ‘skirt’ region of IL-17AF, which appears to be stabilised in a single conformation by the unique heterodimer interface.

As previously noted for IL-17AA, the backbone amide NMR signal of C144 (C152 in IL-17F) is also significantly weaker in both IL-17FF and IL-17AF, however, it is unclear whether this residue is in conformational exchange. In the case of IL-17FF, two adjacent residues (V150 and G151) are also characterised by weak/broad backbone amide signals, which suggests that a small region just above the cysteine knot of IL-17FF is in conformational exchange.

A few very sharp/intense backbone amide signals were also identified in the N- and C-terminal regions of the IL-17 dimers, which is indicative of these regions being highly mobile (Figure 3). The locations of these sharp signals are in close agreement with the regions of the dimers that have previously been shown to be missing from the electron density maps used to determine the published crystal structures, which is also indicative of these regions being dynamic (Hymowitz et al. 2001, Liu et al. 2013, Goepfert et al. 2017). Noticeably fewer sharp/intense signals were identified in the N-terminal region of IL-17FF than in the IL-17F protomer of IL-17AF, suggesting that whilst the unique heterodimer interface appears to stabilise most of the skirt region of IL-17AF, it does not stabilise the extreme N-terminus. Interestingly, a small group of residues near the N-terminus of IL17AA (residues T26-I27), IL-17FF (residues K35-G37) and IL-17AF (IL-17A T26) give rise to two separate sharp backbone amide signals, suggesting that as well as being highly dynamic this region is present in two distinct conformations. In the case of IL-17AA, these highly mobile residues form part of a longer stretch of residues present in two forms.

*3.4 Structural Changes Induced in IL-17 Dimers Upon Receptor Binding*

Crystal structures of IL-17AA, IL-17FF and IL-17AF in complex with IL-17RA, as well as IL-17FF in complex with IL-17RC have been reported (Hymowitz et al. 2001, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017, Goepfert et al. 2020). Despite having two potential symmetrical binding surfaces, the IL-17 homodimers have been shown to form only 1:1 complexes with the D1 and D2 domains of IL-17RA (Figure 5E) (Ely et al. 2009, Liu et al. 2013). Similarly, IL-17AF has been shown to form a 1:1 complex with IL-17RA (Goepfert et al. 2017). It has been proposed that structural changes induced in the skirt region of IL-17 dimers upon IL-17RA binding inhibit the binding of a second IL-17RA molecule (Liu et al. 2013, Goepfert et al. 2017). Analysis of the complex structures shows that the D1 and D2 domains of IL-17RA bind across the dimer interface contacting the IL-17 dimers at three main sites (Figure 5E) (Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017). The D1 domain has been shown to contact both the ‘skirt’ region of one IL-17 protomer (site 1) and a hydrophobic pocket (site 2) located at the dimer interface. The D2 domain interacts with part of the long flexible coil region of the IL-17 subunit that contains the main site 1 and site 2 contact surfaces, as well as contacting the ‘collar’ and C-terminal regions of the other subunit (site 3). Surprisingly, despite the significantly tighter Kd exhibited by IL-17AA for IL-17RA, the available crystal structure shows IL-17RA binding to the predominantly IL-17F face of IL-17AF (Ely et al. 2009, Goepfert et al. 2017). However, SPR studies using both wild-type and mutant IL-17AF suggest that IL-17RA is able to bind to either face of the heterodimer with similar affinities (Goepfert et al. 2017).

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**Figure 5: Comparison of Structural Changes Induced in IL-17AA, IL-17AF and IL-17FF by binding of IL-17RA and IL-17RC.** Panel A shows an overlay of the backbone topology of free (blue, PDB code 1JPY) and IL-17RA-bound (cyan, PDB code 3JVF) IL-17FF. The structures were overlaid on the C⍺ atoms of residues that form the highly conserved cysteine knot region. Panels B and C show equivalent overlays for the free (PDB code 4HR9) and IL-17RA-bound (PDB code 4HSA) IL-17AA (B) and for free (PDB code 5N92) and IL-17RA-bound (PDB code 5NAN) IL-17AF (C). In panel B the structure of free IL-17AA is shown in red, with the IL-17RA bound form in pink. In panel C the IL-17A and IL-17F subunits of free IL-17AF are shown in red and blue respectively, while the IL-17A and IL-17F chains of the IL-17RA-bound heterodimer are shown in pink and cyan respectively. Panel D shows an equivalent overlay of free (blue, PDB code 1JPY) and IL-17RC-bound (magenta, PDB code 6HG4) IL-17FF. Panels E and F illustrate the positioning of IL-17RA (D) and IL-17RC (E) on IL-17FF (PDB codes 3JVF and 6HG4), with IL17FF shown in the same orientation as panel A. In panel E IL-17FF is shown in cyan and the bound D1-D2 domains of IL-17RA in green. Similarly, in panel F IL-17FF is shown in magenta and the two bound molecules of IL-17RC D1-D2 in pale green and yellow. The IL-17RC D3-D4 domains, which do not contact IL17FF, are omitted from the view shown.

Comparison of the published crystals structures of IL-17AA, IL-17AF and IL-17FF reveals that a number of significant structural changes are induced in the dimers upon binding to IL-17RA (Figure 5A-C and Supplementary Figure 3) (Hymowitz et al. 2001, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017). Most notably, changes in the orientation of one of the long coil regions spanning the central dimer interface is required to accommodate binding of the receptor. The interaction of IL-17RA with IL-17 dimers also induces major structural changes in the predominantly unstructured N-terminal region and the β0 sheet, as well as the formation of an additional β-strand in the unstructured C-terminal region of one of the protomers. In order to quantify and further analyse the changes induced upon binding, the structures of the apo- and IL-17RA bound IL-17 dimers were overlaid in PyMOL (Schrodinger) on the Cα atoms of the residues that form the highly conserved cysteine knot (residues C94, C99, C144 and C146 of IL-17A and C102, C107, C152 and C154 or IL-17F), and the Cα-RMSD per residue was calculated using VMD (Figure 3A-D) (Humphrey et al. 1996). In addition to the changes already noted in the flexible coil, ‘skirt’ and C-terminal regions, analysis of the Cα-RMSD plots for IL-17AA highlighted a significant twist in the β1-β2 and β3-β4 turns, and adjacent residues, relative to the cysteine knot upon binding IL-17RA (Figures 3A, 5B and Supplementary Figure 3D). In addition, a significant change in the relative orientation of the β3-β4 turn was induced in the IL-17A subunit that contacts IL-17RA D1.

Comparison of the Cα-RMSDs and the backbone amide peak intensities observed for each residue in IL-17AA revealed that the largest structural changes were observed in regions of IL-17AA where either very weak/broad or no NMR signals were detected (Figure 3A). This broadening and in some cases loss of NMR signals indicates that these regions of IL-17AA interconvert between at least two conformational states. Conformational heterogeneity has previously been shown to be an important feature of many high affinity protein-protein binding sites (Williamson *et al.* 1997, Gao *et al.* 2000, Waters et al. 2007, Veverka *et al.* 2009, Addis *et al.* 2014). It is highly likely that the heterogeneity observed in these regions of IL-17AA plays an important role in accommodating the binding of IL-17RA, resulting in the formation of a tight complex (Kd = 0.7-2.8 nM) (Wright et al. 2008, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017).

The IL-17AF heterodimer has two non-identical potential binding sites for IL-17RA, both of which are believed to be functionally relevant (Goepfert et al. 2017). Only the structure of IL-17RA in contact with the predominantly IL-17F face of the heterodimer has been determined, as a result we are only able to assess the structural changes induced by binding to this site (Goepfert et al. 2017). Analysis of the Cα-RMSD plots for IL-17AF highlighted the previously discussed changes in the unstructured N-terminal regions, β0 sheet, long coil region, and C-terminal region (Figures 3B, 3D, 5C and Supplementary Figure 3B and E). As seen for IL-17AA, there was also an IL-17RA binding induced change in the relative orientation of the β1-β2 and β3-β4 turns, and adjacent residues (Supplementary Figure 3B and E). In contrast to IL-17AA, no change in the orientation of the β3-β4 turn of the IL-17F subunit relative to the adjacent β-sheet is required to accommodate IL-17RA binding to the heterodimer (Supplementary Figure 3B).

Very few broad or missing NMR signals were detected in IL-17AF, suggesting that significantly less conformational exchange is present in the heterodimer (Figures 3B, 3D and 4C). As a result, there is likely to be a more significant energy penalty for the structural changes induced by IL-17RA binding to IL-17AF, than seen for IL-17AA, which may help to explain the weaker Kd observed for the IL-17AF/ IL-17RA complex (Kd=17.5-25.5 nM) (Wright et al. 2008, Goepfert et al. 2017).

Equivalent analysis of the Cα-RMSD plots for IL-17FF highlighted similar structural changes induced by IL-17RA binding to those previously noted for IL-17AA and IL-17AF, including changes in β0, the long coil region, the two β-hairpins (β1-β2 and β3-β4), and C-terminal region (Figures 3C, 5A and Supplementary Figure 3A). The vast majority of the broad/missing NMR signals for IL-17FF are located in the skirt region, where many of the previously mentioned structural changes are found. (Figures 3C, 4B and 5A). However, in contrast to IL-17AA, there is very limited evidence of conformational exchange in the long coil region or the first β-hairpin (Figures 3A, 3C, 4A and 4B). Interestingly, a significantly larger section of the N-terminal region preceding β0 is visible in the crystal structure of apo-IL-17FF than was seen in either the IL-17FF/IL-17RA complex or IL-17AF (Figure 5). This region partially blocks the IL-17RA binding site, and therefore is required to undergo a large structural change to accommodate the binding of IL-17RA. Analysis of the peak intensities observed in the 15N/1H HSQC spectra for this region suggests that it is significantly less dynamic in IL-17FF than in IL-17AF (Figure 3C and 3D). As noted for IL-17AF, it is highly likely that the energetic penalty required to re-orientate the long coil region and the two β-hairpins, probably in combination with the penalty required to destabilise the N-terminal region, significantly contributes to the far weaker Kd observed for the interaction of IL-17RA with IL-17FF (Kd=136-292 nM) (Wright et al. 2008, Ely et al. 2009, Goepfert et al. 2017).

Despite limited sequence conservation between IL-17RA and IL-17RC, both receptors have been shown to contact IL-17FF via equivalent structural motifs (Figure 5E and 5F) (Ely et al. 2009, Goepfert et al. 2020). Interestingly, the binding of IL-17RC was found to induce far fewer structural changes in IL-17FF, allowing the formation of a symmetrical 2:1 IL-17RC:IL17FF complex (Goepfert et al. 2020). Previous SPR-based studies have reported that IL-17AA and IL-17AF are able to bind a single IL-17RC molecule with high affinity, as seen for IL-17RA, however, very recent ITC and biophysical studies suggest that two copies of IL-17RC can interact with single IL-17AA and IL-17AF dimers under some conditions (Ely et al. 2009, Goepfert et al. 2017, Goepfert et al. 2020).

Comparison of the free and receptor bound structures of IL-17FF, combined with analysis of the related Cα-RMSD plots, indicates that very similar structural changes were induced in the N-terminal region that precedes β0, the long coil region and C-terminal region of IL-17FF upon binding both IL-17RA and IL-17RC (Figures 3C, 3E, 5A, 5D and Supplementary Figure 3A and 3C). However, in contrast to the IL-17FF/IL-17RA complex, either no or significantly smaller changes were observed in the orientation of the β-hairpins upon binding IL-17RC (Figures 3C, 3E, 5A, 5D and Supplementary Figure 3A and 3C). As previously discussed for IL-17RA binding, it is likely that the energetic penalty required to re-orientate the N-terminal, extended coil and β3-β4 turn regions of IL-17FF on IL-17RC binding will reduce the affinity of the interaction, however, this may be required to allow rapid switching between signalling.

Previous SPR studies have shown that IL-17RA binds to functional IL-17 dimers with markedly different affinities (Wright et al. 2008, Ely et al. 2009, Goepfert et al. 2017). The tightest interaction is seen with IL-17AA (Kd 0.7-2.8 nM), which binds over an order of magnitude tighter than IL-17AF (Kd 17.5-25.5 nM) and two orders of magnitude tighter than IL-17FF (Kd 136-292 nM) (Wright et al. 2008, Ely et al. 2009, Goepfert et al. 2017). In contrast, the same studies reported that very similar affinities were observed for IL-17RC binding to IL-17AA (Kd 0.4-20.1 nM), IL-17AF (Kd 0.6-10.6 nM) and IL-17FF (Kd 3.5-16.6 nM). Interestingly, the affinity of the interaction of IL-17FF with IL-17RC was less than an order of magnitude weaker than was observed for IL-17AA/IL-17RA. The reported on-rates for the IL-17 dimer /receptor complexes suggest that the association of IL-17RA with IL-17AA (1.39x105 M/s) and IL-17RC with IL-17AA (8.92x104 M/s), IL-17AF (1.44x105 M/s) and IL-17FF (1.28x105 M/s) are all diffusion limited (Wright et al. 2008). The significantly slower on-rates observed for IL-17RA binding to both IL-17AF (4.28x104 M/s) and IL-17FF (9.43x103 M/s) suggests that their association is not diffusion limited, and is consistant with the large number of structural changes required to accommodate the binding of IL-17AF and IL-17FF to IL-17RA (Wright et al. 2008). Given the noticeably lower number of structural changes observed in IL-17FF upon binding IL-17RC and the similar on-rates and affinities observed for all three IL-17 dimers binding to IL-17RC, it would appear that the very different dynamic properties of the IL-17 functional dimers play a far less important role in determining the affinity of binding to IL-17RC compared to IL-17RA.

The NMR studies reported here revealed considerably less conformational exchange in the β-hairpins of each subunit of IL-17FF and IL-17AF compared to IL-17AA (Figure 4). Whilst the β-hairpins of IL-17FF undergo significant structural changes upon binding of IL-17RA only very limited changes are seen here upon binding of IL-17RC (Supplementary Figure 3A and 3C). The strikingly different affinities observed for IL-17FF binding to IL-17RA and IL-17RC strongly suggest that the structural changes induced in the non-dynamic β-hairpin regions of IL-17FF upon binding of IL-17RA introduce a significant energetic penalty resulting in a lower affinity interaction. To date no structure has been reported for the IL-17AF-IL-17RC complex, however, similar reasoning could explain the significantly different affinities observed for IL17AF binding to IL-17RA and IL-17RC.

1. **Discussion**

The proinflammatory cytokines IL-17AA, IL-17AF and IL-17FF play an important role in the immune response (Veldhoen 2017). They are also fully validated targets for the treatment of major inflammatory conditions such as psoriasis and arthritis (Griffiths et al. 2015, Thaci et al. 2015). Consequently, there is considerable ongoing interest in understanding the molecular mechanisms by which these dimeric cytokines function. IL-17AA, IL-17FF and IL-17AF have all been shown to induce signalling via the formation of a ternary complex with IL-17RA and IL-17RC (Toy *et al.* 2006, Wright et al. 2008, Hu *et al.* 2010). The affinity of IL-17RA for the three distinct IL-17 functional dimers has been found to differ very markedly, with binding to IL-17FF being approximately 100 fold weaker compared to IL-17AA (Wright et al. 2008, Ely et al. 2009, Goepfert et al. 2017). The functional significance and molecular basis for these striking differences in the affinity of IL-17RA binding remain unclear. It has previously been suggested that non-conserved side chain interactions at the IL-17RA contact surfaces might account for the different binding affinities (Liu et al. 2013). However, structure-guided mutational studies of IL-17AA revealed only modest changes in affinity (1.5-5 fold) when selected non-conserved residues were individually substituted by the equivalent residue in IL-17FF (Liu et al. 2013), suggesting that additional factors may also be important. Similarly, studies of the interaction of IL-17AF with IL-17RA and IL-17RC revealed that both receptors are able to bind to either face of the heterodimer with comparable affinities (Goepfert et al. 2017), further highlighting that differences beyond non-conserved side chains at the interfaces play a major role in determining the affinity of IL-17-receptor complex formation.

Careful analysis and comparison of the peak widths and intensities observed for backbone amide NMR signals from IL-17AA, IL-17AF and IL-17FF clearly indicates that significant portions of the ‘skirt’, ‘body’ ‘sleeves’ and long interface coil region of IL-17AA exist in multiple conformations in solution, which are interconverting on a relatively slow timescale. In contrast, this conformational dynamics is largely absent from IL-17FF and IL-17AF. The regions of IL-17AA characterised by this conformational plasticity largely correspond to the areas of the dimer required to undergo significant structural changes to accommodate binding of IL-17RA. Conformational heterogeneity and associated slow motions are believed to play an important role in the formation of many high-affinity protein-protein complexes, which are involved in a diverse range of biological processes (Williamson et al. 1997, Gao et al. 2000, Waters et al. 2007, Veverka et al. 2009, Addis et al. 2014). This behaviour could serve many functions, including acting as a ‘soft capture, protein-protein docking mechanism’, or allowing structurally diverse binding partners to compete for binding to a common binding surface (Addis et al. 2014). In the case of IL-17AA, it is probable that the affected regions are either sampling conformations similar to those observed in the receptor-bound complexes, or that conformational plasticity makes it easier to adopt the required conformation upon binding. As highlighted above, noticeably less conformational exchange was detected in IL-17FF and IL-17AF. As a result, the structural changes required in IL-17FF and IL-17AF to accommodate IL-17RA binding are expected to incur a higher energy penalty, which is likely to be a major contributor to the weaker affinities seen for IL-17RA compared to IL-17AA.

In contrast to the wide range of affinities observed for IL-17RA binding to IL-17 family dimers, significantly less variation was found for binding to IL-17RC (Wright et al. 2008, Ely et al. 2009, Goepfert et al. 2017). As a consequence, IL-17AF and IL-17FF bind to IL-17RC over an order of magnitude more tightly than IL-17RA (Wright et al. 2008, Ely et al. 2009, Goepfert et al. 2017). Comparison of the available crystal structures showed that binding of IL-17RC induced fewer structural changes in IL-17FF than seen for binding to IL-17RA (Hymowitz et al. 2001, Ely et al. 2009, Goepfert et al. 2020). This strongly suggests that the differing conformational plasticity identified for IL-17 family dimers in solution will have a far smaller influence on the affinity of binding to IL-17RC compared to IL-17RA.

As highlighted above, significant and different structural changes are induced in IL-17 family dimers upon binding their receptors (Hymowitz et al. 2001, Ely et al. 2009, Liu et al. 2013, Goepfert et al. 2017, Goepfert et al. 2020). Importantly, the changes induced by IL-17RA binding have been proposed to result in structural asymmetry of IL-17 dimers that significantly inhibits binding of a second IL-17RA molecule and favours the formation of an IL-17RC containing signalling complex (Goepfert et al. 2017). The NMR work reported here provides the first key insights into the importance of conformational plasticity in governing the affinity of IL-17 family dimers for IL-17RA. In addition, the binding of an initial receptor molecule to an IL-17 dimer is expected to reduce the extent of conformational exchange present, as indicated by HDX-MS studies comparing apo- and IL-17RA-bound IL-17AA, which showed that most regions of IL-17AA exhibited protection to deuterium exchange upon binding IL-17RA (Espada et al. 2016). Whilst the protection was attributed to structural changes, it seems highly likely that the suggested reduction in conformational exchange observed upon receptor binding would also contribute to the decreased rate of deuterium uptake. This decrease in conformational plasticity is likely to contribute to the substantially reduced affinity seen for binding of a second receptor molecule.

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**References**

Adams, R., Maroof, A., Baker, T., Lawson, A. D. G. Oliver, R., Paveley, R., Rapecki, S., Shaw, S., Vajjah, P., West, S. and Griffiths, M. (2020). "Bimekizumab, a Novel Humanized IgG1 Antibody that Neutralizes both IL-17A and IL-17F." Front. Immunol. DOI: 10.3389/fimmu.2020.01894

Addis, P. W., C. J. Hall, S. Bruton, V. Veverka, I. C. Wilkinson, F. W. Muskett, P. S. Renshaw, C. E. Prosser, B. Carrington, A. D. Lawson, R. Griffin, R. J. Taylor, L. C. Waters, A. J. Henry and M. D. Carr (2014). "Conformational heterogeneity in antibody-protein antigen recognition: implications for high affinity protein complex formation." J Biol Chem **289**(10): 7200-7210.

Bodenhausen, G. and D. J. Ruben (1980). "Natural abundance N-15 NMR by enhanced heteronuclear spectroscopy." Chem. Phys. Lett. **69**(1): 185-189.

Cavanagh, J., W. J. Fairbrother, A. G. Palmer, M. Rance and N. J. Skelton (2007). Chemical Exchange Effects in NMR Spectroscopy. Protein NMR Spectroscopy Elsevier Academic Press**:** 391-400.

Chang, S. H. and C. Dong (2007). "A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses." Cell Res **17**(5): 435-440.

Delaglio, F. "Non-Uniform Sampling in NMRPipe." Retrieved 02/10/2020, from <https://www.ibbr.umd.edu/nmrpipe/nus.html>.

Delaglio, F., S. Grzesiek, G. Vuister, G. Zhu, J. Pfeifer and A. Bax (1995). "NMRPIPE - A multidimensional spectral processing system based on UNIX pipes." J. Biomol. NMR **6**(3): 277-293.

Ely, L. K., S. Fischer and K. C. Garcia (2009). "Structural basis of receptor sharing by interleukin 17 cytokines." Nat Immunol **10**(12): 1245-1251.

Espada, A., H. Broughton, S. Jones, M. J. Chalmers and J. A. Dodge (2016). "A Binding Site on IL-17A for Inhibitory Macrocycles Revealed by Hydrogen/Deuterium Exchange Mass Spectrometry." J Med Chem **59**(5): 2255-2260.

Gaffen, S. L. (2009). "Structure and signalling in the IL-17 receptor family." Nat Rev Immunol **9**(8): 556-567.

Gao, G., V. Semenchenko, S. Arumugam and S. R. Van Doren (2000). "Tissue inhibitor of metalloproteinases-1 undergoes microsecond to millisecond motions at sites of matrix metalloproteinase-induced fit." J Mol Biol **301**(2): 537-552.

Gerhardt, S., W. M. Abbott, D. Hargreaves, R. A. Pauptit, R. A. Davies, M. R. Needham, C. Langham, W. Barker, A. Aziz, M. J. Snow, S. Dawson, F. Welsh, T. Wilkinson, T. Vaugan, G. Beste, S. Bishop, B. Popovic, G. Rees, M. Sleeman, S. J. Tuske, S. J. Coales, Y. Hamuro and C. Russell (2009). "Structure of IL-17A in complex with a potent, fully human neutralizing antibody." J Mol Biol **394**(5): 905-921.

Glatt, S., D. Baeten, T. Baker, M. Griffiths, L. Ionescu, A. D. G. Lawson, A. Maroof, R. Oliver, S. Popa, F. Strimenopoulou, P. Vajjah, M. I. L. Watling, N. Yeremenko, P. Miossec and S. Shaw (2018). "Dual IL-17A and IL-17F neutralisation by bimekizumab in psoriatic arthritis: evidence from preclinical experiments and a randomised placebo-controlled clinical trial that IL-17F contributes to human chronic tissue inflammation." Ann Rheum Dis **77**(4): 523-532.

Goddard, T. D., D. G. Kneller and (unpublished). "SPARKY 3." University of California, San Francisco: <http://www.cgl.ucsf.edu/home/sparky/>.

Goepfert, A., S. Lehmann, J. Blank, F. Kolbinger and J. M. Rondeau (2020). "Structural Analysis Reveals that the Cytokine IL-17F Forms a Homodimeric Complex with Receptor IL-17RC to Drive IL-17RA-Independent Signaling." Immunity **52**(3): 499-512 e495.

Goepfert, A., S. Lehmann, E. Wirth and J. M. Rondeau (2017). "The human IL-17A/F heterodimer: a two-faced cytokine with unique receptor recognition properties." Sci Rep **7**(1): 8906.

Griffiths, C. E., K. Reich, M. Lebwohl, P. van de Kerkhof, C. Paul, A. Menter, G. S. Cameron, J. Erickson, L. Zhang, R. J. Secrest, S. Ball, D. K. Braun, O. O. Osuntokun, M. P. Heffernan, B. J. Nickoloff, K. Papp, Uncover and U.-. investigators (2015). "Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two phase 3 randomised trials." Lancet **386**(9993): 541-551.

Grzesiek, S. and A. Bax (1992). "Improved 3D triple-resonance NMR techniques applied to a 31-kDa protein." J. Magn. Reson. **96**(2): 432-440.

Hu, Y., N. Ota, I. Peng, C. J. Refino, D. M. Danilenko, P. Caplazi and W. Ouyang (2010). "IL-17RC is required for IL-17A- and IL-17F-dependent signaling and the pathogenesis of experimental autoimmune encephalomyelitis." J Immunol **184**(8): 4307-4316.

Humphrey, W., A. Dalke and K. Schulten (1996). "VMD: visual molecular dynamics." J Mol Graph **14**(1): 33-38, 27-38.

Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, G. Pan, A. L. Gurney, A. M. de Vos and M. A. Starovasnik (2001). "IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding." EMBO J **20**(19): 5332-5341.

Karunairatnam, M. C., J. Spizizen and H. Gest (1958). "Preparation and properties of protoplasts of Rhodospirillum rubrum." Biochim Biophys Acta **29**(3): 649-650.

Lee, W., M. Tonelli and J. L. Markley (2015). "NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy." Bioinformatics **31**(8): 1325-1327.

Liu, S., X. Song, B. A. Chrunyk, S. Shanker, L. R. Hoth, E. S. Marr and M. C. Griffor (2013). "Crystal structures of interleukin 17A and its complex with IL-17 receptor A." Nat Commun **4**: 1888.

Marintchev, A., D. Frueh and G. Wagner (2007). "NMR methods for studying protein-protein interactions involved in translation initiation." Methods Enzymol **430**: 283-331.

Marion, D., L. E. Kay, S. W. Sparks, D. A. Torchia and A. Bax (1989). "3-Dimensional heteronuclear NMR of N-15-labeled proteins." J. Am. Chem. Soc. **111**(4): 1515-1517.

Morin, A., B. Eisenbraun, J. Key, P. C. Sanschagrin, M. A. Timony, M. Ottaviano and P. Sliz (2013). "Collaboration gets the most out of software." Elife **2**: e01456.

Pervushin, K. V., G. Wider and K. Wuthrich (1998). "Single Transition-to-single Transition Polarization Transfer (ST2-PT) in [15N,1H]-TROSY." J Biomol NMR **12**(2): 345-348.

Salzmann, M., G. Wider, K. Pervushin, H. Senn and K. Wuthrich (1999). "TROSY-type Triple-Resonance Experiments for Sequential NMR Assignments of Large Proteins." J Am Chem Soc **121**: 844-848.

Schett, G., D. Elewaut, I. B. McInnes, J. M. Dayer and M. F. Neurath (2013). "How cytokine networks fuel inflammation: Toward a cytokine-based disease taxonomy." Nat Med **19**(7): 822-824.

Shen, Y. and A. Bax (2013). "Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks." J Biomol NMR **56**(3): 227-241.

Thaci, D., A. Blauvelt, K. Reich, T. F. Tsai, F. Vanaclocha, K. Kingo, M. Ziv, A. Pinter, S. Hugot, R. You and M. Milutinovic (2015). "Secukinumab is superior to ustekinumab in clearing skin of subjects with moderate to severe plaque psoriasis: CLEAR, a randomized controlled trial." J Am Acad Dermatol **73**(3): 400-409.

Toy, D., D. Kugler, M. Wolfson, T. Vanden Bos, J. Gurgel, J. Derry, J. Tocker and J. Peschon (2006). "Cutting edge: interleukin 17 signals through a heteromeric receptor complex." J Immunol **177**(1): 36-39.

Veldhoen, M. (2017). "Interleukin 17 is a chief orchestrator of immunity." Nat Immunol **18**(6): 612-621.

Veverka, V., A. J. Henry, P. M. Slocombe, A. Ventom, B. Mulloy, F. W. Muskett, M. Muzylak, K. Greenslade, A. Moore, L. Zhang, J. Gong, X. Qian, C. Paszty, R. J. Taylor, M. K. Robinson and M. D. Carr (2009). "Characterization of the structural features and interactions of sclerostin: molecular insight into a key regulator of Wnt-mediated bone formation." J Biol Chem **284**(16): 10890-10900.

Waters, L. C., V. Veverka, M. Bohm, T. Schmedt, P. T. Choong, F. W. Muskett, K. H. Klempnauer and M. D. Carr (2007). "Structure of the C-terminal MA-3 domain of the tumour suppressor protein Pdcd4 and characterisation of its interaction with eIF4A." Oncogene **26**(34): 4941-4950.

Williamson, R. A., M. D. Carr, T. A. Frenkiel, J. Feeney and R. B. Freedman (1997). "Mapping the binding site for matrix metalloproteinase on the N-terminal domain of the tissue inhibitor of metalloproteinases-2 by NMR chemical shift perturbation." Biochemistry **36**(45): 13882-13889.

Wittekind, M. and L. Mueller (1993). "HNCACB, a high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with the alpha-carbon and beta-carbon resonances in proteins." J. Magn. Reson. B. **101**(2): 201-205.

Wright, J. F., F. Bennett, B. Li, J. Brooks, D. P. Luxenberg, M. J. Whitters, K. N. Tomkinson, L. J. Fitz, N. M. Wolfman, M. Collins, K. Dunussi-Joannopoulos, M. Chatterjee-Kishore and B. M. Carreno (2008). "The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex." J Immunol **181**(4): 2799-2805.