Structural basis of activation and antagonism of receptor signaling mediated by interleukin-27

Highlights
- Mouse and human IL-27 adopt highly similar structures
- IL-27Rα interacts both with the p28 and EBI3 subunits of IL-27
- SRF388 and IL-27Rα occupy mutually exclusive binding sites on IL-27
- IL-27 mediates receptor assemblies distinct from IL-12 and IL-23

Authors
Katarzyna Składanowska, Yehudi Bloch, Jamie Strand, ..., Christopher A. Hunter, Jonathan A. Hill, Savvas N. Savvides

Correspondence
jhill@surfaceoncology.com (J.A.H.), savvas.savvides@ugent.be (S.N.S.)

In brief
Interleukin-27 (IL-27) activates its cognate receptors on cells to regulate innate and adaptive immune responses in T cell populations, an important area for basic and applied biomedicine. Składanowska and Bloch et al. report the structure and mechanism by which IL-27 binds its signaling receptors and show how a therapeutic antibody antagonizes IL-27 activity.
Structural basis of activation and antagonism of receptor signaling mediated by interleukin-27

Katarzyna Skladanowska,1,2,14 Yehudi Bloch,1,2,14 Jamie Strand,3,9 Kerry F. White,3,10 Jing Hua,3,11 Daniel Aldridge,4 Martin Welin,5 Derek T. Logan,6 Arne Soete,6,7 Romain Merceron,1,2,12 Casey Murphy,1,2,12 Mathias Provost,1,2 J. Fernando Bazan,2,8 Christopher A. Hunter,4 Jonathan A. Hill,3,15 and Savvas N. Savvides1,2,15,*

1Unit for Structural Biology, Department of Biochemistry and Microbiology Ghent University, Technologiepark 71, 9052 Ghent, Belgium
2Unit for Structural Biology, VIB-UGent Center for Inflammation Research, Technologiepark 71, 9052 Ghent, Belgium
3Surface Oncology, 50 Hampshire Street, Cambridge, MA 02139, USA
4Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA
5SARomics Biostructures AB, Medicon Village, Scheelevägen 2, 223 63 Lund, Sweden
6Department of Biomedical Molecular Biology, Faculty of Science, Ghent University, Ghent, Belgium
7Data Mining and Modeling for Biomedicine, VIB-UGent Center for Inflammation Research, Ghent, Belgium
8Bioconsulting, Stillwater, MN, USA
9Present address: NextPoint Therapeutics, 450 Kendall Street, Cambridge, MA 02142, USA
10Present address: Phenomic AI, 661 University Avenue, Toronto, ON M5G0B7, Canada
11Present address: Novartis Institute for Biomedical Research (NIBR), Cambridge, MA, USA
12Present address: Eurofins DiscoverX Products France (EDPF), Le BoisL’Éveque, 86600 Celle-Lévescault, France
13Present address: Aelin Therapeutics, Gaston Geenslaan 1, 3001 Leuven, Belgium
14These authors contributed equally
15Lead contact
*Correspondence: jhill@surfaceoncology.com (J.A.H.), savvas.savvides@ugent.be (S.N.S.)
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SUMMARY

Interleukin-27 (IL-27) uniquely assembles p28 and EBI3 subunits to a heterodimeric cytokine that signals via IL-27Rα and gp130. To provide the structural framework for receptor activation by IL-27 and its emerging therapeutic targeting, we report here crystal structures of mouse IL-27 in complex with IL-27Rα and of human IL-27 in complex with SRF388, a monoclonal antibody undergoing clinical trials with oncology indications. One face of the helical p28 subunit interacts with EBI3, while the opposite face nestles into the interdomain elbow of IL-27Rα to juxtapose IL-27Rα to EBI3. This orients IL-27Rα for paired signaling with gp130, which only uses its immunoglobulin domain to bind to IL-27. Such a signaling complex is distinct from those mediated by IL-12 and IL-23. The SRF388 binding epitope on IL-27 overlaps with the IL-27Rα interaction site explaining its potent antagonistic properties. Collectively, our findings will facilitate the mechanistic interrogation, engineering, and therapeutic targeting of IL-27.

INTRODUCTION

Interleukin-12 (IL-12) family cytokines (IL-12, IL-23, and IL-27, and the more recently reported IL-35 and IL-39) are distinguished by the pairing of their helical IL-6-like cytokine subunits (α-subunit) with soluble receptor chains (β-subunit), and the subsequent sharing of signaling receptors that regulate innate and adaptive immune responses in T cell populations (Hasegawa et al., 2016; Pflanz et al., 2004). IL-27 is produced by activated antigen-presenting cells, such as dendritic cells and activated macrophages, and has emerged as perhaps the most unique member of the IL-12 family. IL-27 comprises a heterodimeric assembly of a p28 helical cytokine subunit with the compact soluble receptor Epstein-Barr virus-induced gene 3 (EBI3), respectively serving as the α- and β-cytokine subunits of a non-covalently linked heterodimeric cytokine. IL-27 signals through its specific cognate receptor IL-27Rα (also known as WSX-1 or TCCR) and the shared receptor gp130 (Pflanz et al., 2002, 2004) to drive Signal Transducer and Activator of Transcription (STAT) 1 and 3 signaling pathways (Pflanz et al., 2004; Wojno et al., 2019). The predicted structural homology of IL-27 with the archetypical IL-12 and IL-23 composite cytokines (that share a common p40 soluble receptor β-subunit), and the similarity of the p28 cytokine α-subunit with IL-6, imparted a pro-inflammatory skew to its ability to promote the production of interferon-γ (IFN-γ) by natural killer (NK) and T cells via Th1 responses. However, the currently understood functional landscape of IL-27 calls for a much broader influence on the inflammation spectrum due to its ability to modify CD4+ and CD8+ T cell effector functions, to promote T regulatory cell responses, and to orchestrate a suppressive transcriptional network (Wojno et al., 2019; Yoshida and Hunter, 2015). For instance, IL-27 is a potent inducer of the anti-inflammatory cytokine IL-10 (Awasthi et al., 2007; Fitzgerald et al., 2007; Stumhofer et al., 2007), which suppresses the development of Th17 cells
limiting autoimmune encephalitis and uveitis (Batten et al., 2006; Chong et al., 2015), and regulating transcriptional networks leading to suppression of inflammation (Zhang et al., 2020).

The immunoregulatory functions of IL-27 and its ability to induce the co-expression of inhibitory receptors (Chihara et al., 2018; De-Long et al., 2018, 2019) have inevitably prompted consideration about its therapeutic targeting (Andrews et al., 2016; Wojno et al., 2019). Most notably, the monoclonal antibody SRF388, a fully human immunoglobulin (Ig)G1 antibody raised against IL-27, is currently being evaluated as a monotherapy and in combination with pembrolizumab in patients with advanced solid tumors in phase 1/1b, open-label, first-in-human, dose-escalation and expansion studies (Patnaik et al., 2021) (ClinicalTrials.gov Identifier: NCT04374877). Inhibition of IL-27 with SRF388 reduces STAT1 phosphorylation leading to increased pro-inflammatory cytokine secretion coupled to anti-tumor readouts and reduced expression of inhibitory immune checkpoint receptors that may result in anticancer therapeutic activity.

Although the pleiotropic immunoregulatory landscape for IL-12 and IL-23, including their role in physiology and disease, have been examined in detail over the past 2 decades (Wojno et al., 2019), our understanding of the structural determinants for the assembly of their promiscuous receptor complexes is incomplete. The current view has been derived from recent structural breakthroughs on complexes mediated by IL-23 and IL-12 (Bloch et al., 2018; Glassman et al., 2021), and structure-driven mechanistic interrogation (Esch et al., 2020; Floss et al., 2020; Georgy et al., 2021). However, it is now clear that this cannot universally apply to the entire family due to fundamental differences in the type and pairing of α- and β-cytokine subunits and the domain organization and pairing of receptors.

Here, we provide the structural basis for how mouse and human IL-27 construct a non-covalent heterodimeric cytokine able to mediate STAT1/STAT3 signaling and how IL-27 recruits the cognate receptor IL-27R to form a classic "site 1" interaction for cytokine receptor binding. Our structural studies of mouse and human IL-27 jointly reveal that heterodimeric IL-27 adopts a highly conserved structure (except for monotreme EBI3) cluster distinctly from those in "site 2"-subunits into a heterodimeric cytokine with ultra-high affinity (Figure 1D). In addition, SRF388 fab, a more liberal secretory behavior (Muller et al., 2019a).

RESULTS

Biochemical reconstitution of IL-27 in complex with IL-27Rα and SRF388 fab for structural studies

To elucidate the structural basis of the unique heterodimeric architecture of IL-27 and its receptor usage (Figure 1A), we pursued structural studies of mouse IL-27 in complex with domains D1 and D2 comprising the cytokine-receptor homology region (CHR) of IL-27Rα (IL-27RαCHR). In parallel we aimed to elucidate the structure of human IL-27 in complex with the Fab fragment of SRF388 (SRF388 fab), a neutralizing monoclonal antibody against IL-27 activity currently in clinical trials with oncology indications.

To date, the production of purified recombinant mouse and human IL-27 (mIL-27 and hIL-27) to amounts that can support biochemical and structural studies proved to be challenging (Detry et al., 2019; Muller et al., 2019b; Pfanz et al., 2002), which prompted the fusion of p28 and EBI3 via a peptide linker in most commercially available mouse and human IL-27 forms. Furthermore, human IL-27p28 critically requires the autonomously expressing EBI3 for secretion, while the mouse orthologues display more liberal secretory behavior (Muller et al., 2019a).

We succeeded in producing recombinant mIL-27 to milligram quantities as a non-covalent glycosylated heterodimeric cytokine by transient co-transfection of its alpha and beta subunits in HEK293 S cells. Such recombinant mIL-27 was equally potent in eliciting STAT1/STAT3 signaling as two distinct fusion cytokine variants comprising the EBI3 subunit fused to the p28 subunit via a (GGGS)₄ linker (see method details) (Figure 1B). Size-exclusion chromatography of such mIL-27 revealed a rather broad elution profile, which became drastically more monodisperse upon formation of a 1:1 stoichiometric complex with IL-27RαCHR (Figure S1A). This suggested that secreting the two proteins in conditioned media as a complex following co-expression in HEK293 S MGAT1−/− cells (Reeves et al., 2002) might provide a good strategy for purifying mIL-27IL-27RαCHR complex amenable to structure determination by X-ray crystallography. Indeed, initial efforts to crystallize this complex following enzymatic shaving of N-linked glycans from purified mIL-27:IL-27RαCHR complex led to crystals, albeit of poor X-ray diffraction quality. However, using a non-neutralizing single-domain camelid antibody fragment (Nb5) raised against the mouse IL-27:IL-27RαCHR:gp130 complex (Figures S2A–S2C) followed by enzymatic shaving of N-linked glycosylation yielded diffraction-quality crystals of the IL-27:IL-27RαCHR:gp130Nb5 complex (Figure 1C) after several rounds of crystal optimization including micro-seeding crystallization strategies (Table 1). In parallel, we confirmed that the Fab fragment of monoclonal antibody SRF388 (SRF388 fab) could bind to recombinant hIL-27 produced in its native format as a non-covalently linked heterodimeric cytokine with ultra-high affinity (Figure 1D). In addition, SRF388 fab, an anti-IL-27 monoclonal antibody currently in clinical trials with oncology indications. Collectively our work classifies the receptor complex mediated by IL-27 separately from IL-12 and IL-23, thereby expanding the structural repertoire within the IL-12 family of cytokines and receptors.

Structure of mouse and human IL-27

Our structural studies of mouse and human IL-27 jointly reveal that heterodimeric IL-27 adopts a highly conserved structure with p28 folding into a long-chain four-helix bundle that nestles into the elbow junction between the two structural modules of EBI3’s CHR (Figures 1E and 1F). Interestingly, phylogenetic analysis of EBI3 revealed that mammalian EBI3 orthologues (except for monotreme EBI3) cluster distinctly from those in evolutionarily distant vertebrates, which possesses an additional N-terminal Ig-like domain (Figure S3). Helix αA and the unique αB'-N-terminal to the long αB helix in IL-27p28 create an interaction interface covering approximately 1,200 Å² that collectively form a classic "site 1" interaction for cytokine receptor binding. While the assembly of IL-27 α- and β-subunits into a heterodimeric cytokine is strongly reminiscent of composite IL-12 and IL-23 cytokines, there are important distinctions. Whereas the respective α-subunits of IL-12 and IL-23 are covalently tethered
Figure 1. Crystal structures of the mouse IL-27 in complex with IL-27Rα and human IL-27 in complex with SRF388Fab.

(A) Overview of the IL-12 family of cytokines and their receptors. Immunoglobulin (Ig) domains are shown as ovals with thick black outline. The cytokine-binding homology region (CHR) consisting of tandem fibronectin type III domains (FNIII) is shown with a double line in the upper domain and a single line in the lower domain. For IL-35 signaling complexes comprising gp130/gp130, IL-12Rβ2/IL-12Rβ2, and IL-27Rα/IL-12Rβ2 have also been described (Pylyayeva-Gupta, 2016).

(B) Comparison of the signaling activity of the recombinant mIL-27 (used for crystallization), recombinant single chain mouse IL-27, mIL-27sc (used for BLI), and commercially produced recombinant single chain mouse IL-27 from BioLegend, mIL-27scBL. STAT1 and STAT3 activity was measured in CD8+ T cells by flow cytometry upon stimulation with increasing concentrations of mIL-27. n = 3 technical replicates/data points, with error bars indicating SEM. These data are representative of two independent experiments with splenocytes from two distinct mice.

(C) Chromatogram of the mIL-27:mIL-27Rα:Nb5 complex from the Superdex200 increase column. Coomassie-stained TGX gel run under denaturing conditions depicts the complex used for the crystallographic trials. Western blot of the same sample with antibodies specific to the respective subunits run in parallel is depicted on the right. Full blot is available in Figure S1B.

(D) Kinetic binding profiles of hIL-27:SRF388 interaction characterized by the BLI. Anti-human IgG Fc (AHC) biosensors were used to immobilize SRF388 followed by binding measurements in different concentrations of hIL-27. One biosensor was used as a reference channel for background subtraction. Data traces (black) were fitted using a 1:1 interaction model (red) to quantify the kinetics ($k_a$, $k_d$) and binding affinity ($K_D$) of the interactions. Data were analyzed with Octet Data Analysis software v10.0.1.7 (ForteBio). Data presented represent one experiment performed using optimized assay conditions. Additional BLI experiments were conducted; however, the $k_d$ exceeded the limit for dissociation. MSD solution-phase studies were used to confirm the BLI measurements (see STAR methods for additional detail).

(E) Cartoon representation of the mIL-27:mIL-27Rα:Nb5 crystal structure. Nanobody 5 used as a crystallization adjuvant is shown in gray surface representation.

(F) Cartoon representation of the hIL-27:SRF388Fab crystal structure. Inhibiting antibody SRF388Fab is shown in surface representation with light chain (LC) in magenta and heavy chain (HC) in light pink. Related to Figures S1–S3.
| Protein                          | Mouse IL-27:IL-27Rx complex | Human IL-27:SRF388Fab complex |
|---------------------------------|-----------------------------|-------------------------------|
| PDB code                        | 7zg0                        | 7zxk                          |
| Crystallization condition       | 1 M Na/K tartrate, 0.1 M MES/NaOH pH 5.5 | 0.2 M ammonium citrate, 20% PEG 3350 |
| Cryoprotectant                  | 4 M sodium formate          | ML + 25% glycerol             |
| Data collection                 |                             |                               |
| Beamline                        | Petra III (P14)             | Diamond (I04)                 |
| Wavelength (Å)                  | 0.9763                      | 0.9795                        |
| Resolution range (Å)            | 79.5–3.18 (3.29–3.18)       | 74.2–3.18 (3.29–3.18)         | 120.4–2.2 (2.26–2.2) | 120.4–2.02 (2.25–2.02) |
| Space group                     | C 2                         | P 2,                          |
| Unit cell                       | a = 167.20 Å, b = 137.67 Å, c = 111.41 Å, α = γ = 90°, β = 117.50 | a = 86.81 Å, b = 104.62 Å, c = 121.75 Å, α = γ = 90°, β = 98.65 |
| Total reflections               | 263419 (26380)              | 199040 (25015)                | 570711 (42769) | 459033 (17211) |
| Unique reflections              | 37419 (3736)                | 33175 (3741)                  | 108947 (8013) | 91590 (4581) |
| Multiplicity                    | 7.0 (7.1)                   | 6.0 (6.7)                     | 5.2 (6.3) | 5.0 (3.8) |
| Completeness (%)                | 99.29 (99.57)               | 87.92 (99.49)                 | 99.8 (99.60) | 95.04 (74.83) |
| Mean I/σ(I)                     | 9.31 (1.12)                 | 7.26 (1.14)                   | 14.82 (0.82) | 16.57 (1.74) |
| Wilson B-factor (Å²)            | 93.71                       | 85.08                         | 59.84 | 45.98 |
| R-meas                          | 0.1892 (1.811)              | 0.2483 (1.763)                | 0.060 (2.081) | 0.053 (0.851) |
| CC ½ (%)                        | 99.6 (41.2)                 | 99.2 (39.6)                   | 99.9 (71.1) | 99.9 (72.6) |
| Refinement                      |                             |                               |
| Reflections for refinement      | 37409 (3736)                | 33161 (3741)                  | 108776 (10764) | 87302 (2349) |
| Reflections (cross-validation)  | 2247 (226)                  | 1982 (226)                    | 5437 (540) | 4363 (119) |
| R-work                          | 0.2535 (0.3390)             | 0.2396 (0.3367)               | 0.1957 (0.3773) | 0.1871 (0.2454) |
| R-free                          | 0.2849 (0.3766)             | 0.2834 (0.3671)               | 0.2262 (0.4027) | 0.2207 (0.2883) |
| No. of non-hydrogen atoms       | 10630                       | 12878                         |
| macromolecules                  | 10525                       | 12284                         |
| ligands                         | 98                          | 28                            |
| solvent                         | 7                           | 566                           |
| Protein residues                | 1330                        | 1575                          |
| RMS(bonds) (Å)                  | 0.002                       | 0.003                         |
| RMS(angles) (°)                 | 0.57                        | 0.59                          |
| Ramachandran favored (%)        | 98.54                       | 97.54                         |
| Ramachandran allowed (%)        | 1.31                        | 2.46                          |
| Ramachandran outliers (%)       | 0.15                        | 0.00                          |
| Rotamer outliers (%)            | 0.00                        | 0.07                          |
| Clash score                     | 4.33                        | 3.19                          |
| Average B-factor (Å²)           | 102.16                      | 61.37                         |
| macromolecules                  | 101.77                      | 61.54                         |
| ligands                         | 148.18                      | 107.51                        |
| solvent                         | 47.93                       | 55.31                         |
| Number of TLS groups            | 12                          | 14                            |

Statistics for the highest-resolution shell are shown in parentheses.

*aCorrected for overlapping spots due to non-merohedral twinning (see STAR methods).

*bCorrected for anisotropy (see STAR methods). Ellipsoidal completeness reported. Refinement statistics are reported to 2.2 Å resolution.
to their common p40 β subunits via their hallmark cysteine disulfide bridges, IL-27 lacks this intermolecular latch. Instead, p28 uses the local positively charged dipole of its short αB0 helix to nestle against a negatively charged patch on EBI3, leading to the projection of two conserved aromatic residues on the same face of αB0 against a hydrophobic patch on EBI3 (Figures 2A–2D). Together, such a binding landscape establishes a nonlinear interaction interface between the p28 and EBI3 subunits of IL-27. Supporting the importance of this unique structural feature of heterodimeric hIL-27, previous studies showed that mutation of W97 in hIL-27p28 and F97 in hEBI3 to alanine severely impacts the secretability of hIL-27 from mammalian cells (Figures S4A, S4B, and S5A) (Rousseau et al., 2010). Finally, the importance of the site 1 interface in the stability and secretability of IL-27 was additionally interrogated by site-directed mutagenesis of positions D210 and E159 in EBI3 (Figure S5A) (Rousseau et al., 2010). Collectively, these structure-function undertakings underpin the functional relevance of the site 1 interaction interface in heterodimeric IL-27.

**IL-27Rα and SRF388 occupy mutually exclusive binding sites on IL-27**

The helical bundle face defined by the αA and αC helices in turn recruits IL-27Rα and SRF388 via site 2 (Figures 1E and 1F). The structure of the IL-27:IL-27RαCHR complex illustrates how the two N-terminal FnIII-type domains of the IL-27Rα CHR engage the AC face of IL-27p28 primarily via αC. In doing so, the D2 domain of IL-27Rα contacts the D2 domain of EBI3 in a stem-like receptor-receptor interaction reminiscent of other hematopoietic receptor complexes (Boulander et al., 2003; de Vos et al., 1992; Verstraete et al., 2014, 2017). We note that Nb5 binds exclusively to EBI3 and mainly contacts D1 of EBI3 via its CDR3 loop, distant from any of the functionally important interfaces of IL-27 (Figures S2D–S2F). Consistent with its antagonistic binding mode against IL-27Rα, SRF388Fab uses its heavy chain to predominantly engage the AC face of IL-27p28, effectively blocking its capture by the cognate receptor interaction site 2 (Figure 1F). Thus, the parallel elucidation of distinct structures of receptor- and antibody-bound IL-27 provides direct validation of the observed binding modes and the functional importance of site 2.

**IL-27 is the nexus of key functional interaction sites**

Our crystal structures provide the structural blueprint for at least four interaction sites relevant for the assembly of receptor complexes mediated by mouse and human IL-27 (Tables S1 and S2). In addition, we provide structural details at high resolution of the binding epitope on hIL-27 exploited by SRF388 to achieve potent antagonism of signaling mediated by hIL-27, providing a structure-based rationale for its therapeutic potential (Tables S1B and S2B).

A structural comparison of mouse and human IL-27 shows that although their overall structures are highly similar (RMSD = 1.16 Å for 310/328 IL-27Cα; RMSD = 1.12 Å for 150/156 p28Cα; RMSD = 1.12 Å for 150/156 EBI3Cα), their respective p28:EBI3 interfaces (site 1) share strong similarities but also differ in significant aspects (Figure 3A). Notably, the conserved Y209mEBI3/Y211hEBI3 interacting with E209mp28/E211hp28, respectively, mark the midpoint of the p28:EBI3 interface (site 1) and are flanked on one side of site 1 by two conserved salt bridges between conserved arginine residues on p28 and...
Figure 3. Comparison of binding interfaces of mouse and human IL-27
(A) Close-up of the site 1 interface in mouse (top) and human (bottom) IL-27.
(B) Close-up of the site 2 interface between mp28 and mL-27Rox (top) and hp28 and SRF388ab.
(C) Sequence of the SRF388ab with CDRs visualized with magenta box (light chain) and light pink box (heavy chain). Residues taking part in site II interface are marked with magenta circles. Sequences were shortened for clarity.
(D) Crystal structure of the mL-27:mL-27Rox:Nb5 binary complex with mp28 in cartoon representation (light blue) and mEBI3 and mL-27Rox in surface electrostatic representation. Structure of the mEBI3 (left) reveals a large positively charged patch on domain 2 at the site of mL-27Rox binding, whereas mL-27Rox possesses a complementary patch that is in turn negatively charged (right).
(E) Close-up view of the mEBI3:mL-27Rox interaction. Binding occurs via a large ~660 Å² interface.
(F) Arginine 51 of the mp28 is interacting simultaneously with E182 of the mEBI3 as well as with Q154 of the mL-27Rox.
(G) Residue R55 from hp28 is observed forming hydrogen bonds with E184 of hEBI3 similarly to the mouse orthologue. Related to Figures S4 and S5, and Tables S1 and S2.
glutamates on EBI3 (Figures 3A and S4). However, the other halves of the site 1 interfaces feature a very divergent interaction scheme, with mp28:EBI3 engaging a trio of polar residues and hp28:EBI3 displaying a rather open structure glued in place by a hydrophobic contact (Figure 3A).

Site 2 interactions at the mIL-27:IL-27Rz interface mainly engage D1 of IL-27Rz that contacts the AC face of IL-27p28 covering a binding footprint of approximately 1,250 Å² (Figures 3B and S4C). Intriguingly, site 2 predominantly features polar interactions and lacks any conspicuous hydrophobic patches (Figures 3B and S4C). We note the engagement of R89 and Y68/K67 on mL-27Rα that reach out to two negatively charged patches on the AC face of mL-27p28, one at E42 on αA and a second at D139/D142 on αC (Figure 3B).

Remarkably, SRF388 binding to IL-27p28 sterically overlaps with site 2 capturing a diverse set of contact residues in αC (Figures 1F, 3B, and S4A) that include the conserved acidic residues key to IL-27Rα binding at site 2 (Figure 3B). The extensive interface evident in the interaction of SRF388 with hIL-27 (Tables S1A and S2B) is consistent with their high affinity and interaction kinetics (Figure 1D). Furthermore, the observed cross-reactivity of SRF388 with human, mouse, rat, and monkey IL-27 orthologues (Figures S1C and S1D) can be traced to the high degree of sequence conservation among the site 2 contact residues across IL-27p28 phylogeny (Figure S4A).

We were surprised by the extent and electrostatic complementarity of contacts mediated by the D2 domains of EBI3 and IL-27Rα CHRs in the mL-27:IL-27Rz complex, accounting for ~660 Å² of buried surface area (Figures 3D and 3E, Table S2A). These “stem region” contacts distinguish IL-27 from the way IL-12 and IL-23 bind to their specific receptors—-with their common IL-12Rβ1 chains recognizing the back face of p40 rather than the respective helical cytokines (Bloch et al., 2018; Glassman et al., 2021)—and instead more closely resemble the IL-6 ternary complex (Boulanger et al., 2003).

We observed a unique contact at the intersection of the tripartite assembly of p28:EBI3:IL-27Rz, where the conserved R51 of mp28 extends downward to interact with E182 of mLEBI3 and Q154 from mL-27Rα (Figure 3F).

Notably, the contact residues in the mL-27:IL-27Rz complex are conserved across the orthologous proteins (Figures 3G, S4B, and S4C), strongly supporting the contribution of these stem region receptor interactions across species. The mechanistic consequences of such an interaction are likely substantial because the alignment of IL-27Rα along D2 of EBI3 will provide directionality and perhaps reduced degrees of freedom to the membrane-proximal domains of IL-27Rα and their possible interactions with the shared receptor gp130.

Finally, neither mL-27 nor hIL-27 in their bound structures presented here provide structural insights into the possible role of the enigmatic poly-glyutamate segments that define the first half of the loop region connecting αC and αD of the p28 subunit (Figures S4 and S5). This is because this entire region is highly disordered in our crystal structures, despite having crystalized mL-27 and hIL-27 in complex with distinct molecular partners and multiple copies of these complexes in the crystal asymmetric units.

High-affinity binding of gp130 to site 3 occurs independently of site 2

Prior studies had proposed that the p28 subunit harbors site 3 for the recruitment of gp130 to the receptor complex mediated by IL-27 and that it can antagonize signaling mediated by gp130 (Rousseau et al., 2010; Stumhofer et al., 2010). Specifically, W195 and W197 in mL-27p28 and hIL-27p28 were projected as candidate hotspots for the binding of the Ig domain of gp130. Indeed, a W195A mutation in mL-27p28 was shown to serve as a potent antagonist against IL-27 in vivo being able to bind IL-27Rz but not gp130. Furthermore, a W197A mutation in hIL-27p28 abrogated STAT3 and STAT1 signaling while retaining the ability to bind to IL-27Rz at the cell surface (Rousseau et al., 2010). Our structural studies on mouse and human IL-27 and complexes with IL-27Rα and SRF388 now provide the structural context of this functional hotspot and show that W195/W197 resides at the N-terminal end of αD and is poised to provide a docking spot for the Ig domain of gp130 (Figure 4A).

To date, quantification of the binding kinetics and affinity of binding for the interaction of IL-27 with cognate receptors had not been reported. To complement our structural findings, we used bio-layer interferometry (BLI) by immobilizing biotinylated mL-27zC, comprising the EBI3 subunit fused to the p28 subunit via a (GGGS)₄ linker (see STAR methods), to a streptavidin biosensor tip. We found that mL-27RzCHR binds to mL-27 with moderately fast and slow on and off rates to yield an equilibrium dissociation constant Kₒ ~ 190 nM (Figure 4C). Interestingly, when we used this high-affinity complex to investigate recruitment of immobilized gp130igCHR we found that binding of the shared receptor displayed a moderately fast on-rate constant similar to the association of mL-27RzCHR, albeit with a surprisingly faster off-rate constant leading to a Kₒ ~ 190 nM (Figure 4C).

Interestingly, binding of gp130 directly to mL-27 in the absence of mL-27RzCHR returns a nearly identical profile of binding kinetics and affinity (Figure 4D). Given the predicted role of the Ig domain of gp130 in binding to IL-27p28 via site 3 (Figure 4A) we interrogated the binding of gp130 lacking its Ig domain to IL-27 and found that the absence of gp130ig abolishes the ability of gp130 to engage IL-27 (Figure 4E). To provide additional insights into the importance of gp130ig in binding to IL-27 we leveraged B-T2, a monoclonal antibody shown to target the Ig domain of gp130 in the context signaling mediated by IL-6 and IL-11 (Kurth et al., 1999). We found that B-T2 inhibited IL-27-dependent pSTAT1 activation of U937 cells and peripheral blood mononuclear cells (PBMC) (Figures 4F and 4G). Together, these data identify the N-terminal Ig domain of gp130 as the mediator for the recruitment of gp130 to IL-27. Importantly, the recruitment of IL-27Rα and gp130 to the signaling complex mediated by IL-27 entails independent binding events, in contrast to the apparent co-operative assembly of the receptor complex mediated by IL-23 (Bloch et al., 2018).

To obtain insights into the structural context of the complete extracellular receptor complex mediated by IL-27 we combined the structural information we reported here with the latest multimer-capable version 2.1 of AlphaFold2 (Evans, 2021) to construct a hybrid model for the complete extracellular complex.
mediated by IL-27 and to compare it with similarly constructed assemblies mediated by IL-23 and IL-6 (Figures 5A–5C). In agreement with our binding studies, gp130 uses only its N-terminal Ig domain to contact mIL-27 via W195 on IL-27p28. In such an assembly, gp130 does not contact the receptor binding domains of IL-27R but displays outward before it swings back to contact the membrane-proximal domain of IL-27R. Such a receptor configuration bears a striking similarity to the ternary receptor complex nucleated by IL-6 (Figure 5C) but is distinct from receptor complexes mediated by IL-23 (Figure 5A). Future studies will need to focus on expanding structural insights from such full-length extracellular assemblies. To this end, recent structural analyses by cryoelectron microscopy at low resolution of minimal ternary receptor assemblies mediated by IL-27 corroborate all structural insights presented in our study (Caveney et al., 2022; Jin et al., 2022).

**DISCUSSION**

The introduction of IL-27 as a composite cytokine with a unique composition, receptor utilization, and functional roles that included both pro- and anti-inflammatory effects dependent on cellular context, drastically expanded the immunological and therapeutic dimensions attributed to the IL-12 family of cytokines (Pflanz et al., 2002, 2004; Wojno et al., 2019; Yoshida and Hunter, 2015). Whereas other members of the IL-12 family with more strongly defined inflammatory effects, such as IL-23 and IL-12, have been intensely pursued for therapeutic applications via antibody and non-antibody approaches (Beyer et al., 2008; Desmet et al., 2014; Desmyter et al., 2017; Luo et al., 2010; Nguyen et al., 2019; Ramamurthy et al., 2012), therapeutic targeting of IL-27 is still in its dawn as the full range of its biology and signaling mechanism is being elucidated.

Two decades after those original discoveries, we provide here the structural blueprint for the IL-27 built from its p28 and EBI3 subunits, and the structural basis for the recruitment of IL-27R and the shared receptor gp130. In parallel, we defined the structural mechanism by which SRF388, a first-in-class monoclonal antibody targeting IL-27 in cancer, neutralizes IL-27 by sterically impeding the binding of IL-27R. Importantly, the structural details generated from our studies are poised to drive drug discovery and protein engineering efforts toward modulating IL-27 stability/secretion and function and the IL-12 family of cytokines in general (Hildenbrand et al., 2022; Muller et al., 2019a, 2019b), and perhaps fine-tuning its pleiotropic immunological effects, analogous to recent advances centered on IL-12/IL-23 (Glassman et al., 2021). For instance, while the free cysteine C107 in hIL-27p28 could indeed be complemented...
by an L162C mutation (Muller et al., 2019b) to create a stabilizing intramolecular disulfide bridge, our structures suggest that F160 might be better suited for mutagenesis to cysteine. In addition, the elucidation of four functional interaction sites mediated by IL-27 (Figures 1E, 1F, 3A, 3B, 3D–3G, and 4A) provides a full array of possibilities for targeting them and their cognate receptor epitopes by a variety of protein and small-molecule-based therapeutic approaches.

Figure 5. The IL-27 receptor assembly is distinct from IL-12/IL-23 complexes and is structurally intermediate between IL-6 and IL-12/23 mediated receptor assemblies

(A) For IL-23, IL-23p19 binds IL-12p40 via site 1; however, IL-23R binds IL-23p19 exclusively with its N-terminal immunoglobulin domain via a site 3 instead of using the elbow region between D2 and D3 as in other type I cytokine receptors. IL-12Rβ1 receptor, which is shared with the IL-12 cytokine, does not engage the classical site 2 but binds p40 at a site opposite to p19, here labeled 2’. Structure of the minimal IL-23 receptor complex (PDB: 6wdo) was supplemented with an AlphaFold model of D2-D5 of IL-12Rβ1.

(B) IL-27 uses the canonical site 1 for the p28:EBI3 interaction as well as site 2 for the p28:IL27Rα binding akin to IL-6:IL-6Rα and IL-6:gp130 binding respectively. gp130 is predicted to bind the conserved W195 in mouse and W197 in human p28. Here the mouse minimal complex presented in this manuscript was combined with the AlphaFold models of D3-D5 of IL-27Rα and a full-length gp130.

(C) IL-6 receptor complex is composed of an IL-6:IL-6Rα:gp130 complex that forms a hexamer. gp130 engages both sites 2 and 3 on IL-6. First it binds site 2 of IL-6 with its elbow region between D2D3 forming an intermediate nonsignaling complex. The resulting complex dimerizes via D1 of gp130 and site 3 of the cytokine on another IL-6:IL-6Rα:gp130 complex. Surface representations of models of the extracellular signaling complexes. Models were assembled from published crystal structures supplemented by AlphaFold2 predictions.
assembly process appears to be devoid or much less dependent on binding cooperativity, in contrast to what has been observed for other tall receptor assemblies such as those mediated by IL-23 (Bloch et al., 2018) and CSF-1 (Eleghheet et al., 2011) for which receptor-receptor contacts contribute critically to the stability of the complexes.

Finally, our structures of mouse and human IL-27 provide templates for mapping and rationalizing common polymorphisms in the genes encoding for m/hIL-27p28 (Figure S5). A common polymorphism in hIL-27p28 is L119P (rs181203) with an allele frequency of 28% (GnomAD) and even higher prevalence in European populations. This residue (M115 in mice) maps to zB in the p28 helical bundle. zB does not contact either EBI3 or IL-27Rα, nor is it expected to mediate binding to gp130. We note that zB in mIL-27p28 can support two prolines while maintaining form and function (Figures S5C–S5E). Thus, the L119P mutein in hIL-27 would not be expected to directly impact function, but rather perhaps affect protein stability and secretion. S59A (rs17855750) and E166D (rs147413292) variants have also been found to be rather common with 6.3% and 4.9% allele frequencies, respectively. S59 (S55 in mice) is located on helix zA facing the solvent (Figure S6E). S59 may indirectly contribute to EBI3 binding via a water-mediated interaction with EBI3 D210 (D208 in mouse EBI3). Interestingly, a D210A mutation in hEBI3 was shown to drastically impact the stability of heterodimeric hIL-27, highlighting the importance of interactions at that position (Rousseau et al., 2010). Last, an E166D variant has been identified that maps to the poly-glutamate stretch linking helices C and D of hIL-27p28 (Figure S5F). This region was not resolved in any of our experimental electron density maps for mouse and human IL-27 and is likely highly flexible. A functional role in bone binding in vivo has been previously proposed for this region (Tormo et al., 2013); however, an E166D mutation would not be expected to affect this function.

Collectively, the findings and insights we report here fill a large void in our current understanding of the pleiotropic cytokine IL-27 and will facilitate its further functional interrogation and therapeutic targeting in physiology and disease.

Limitations of the study
Here, we provided structural and mechanistic insights into the activation and antagonism of IL-27, the latter via a monoclonal antibody undergoing clinical trials with oncology indications. Our crystal structures of mouse and human IL-27 do not provide experimental evidence to support a likely structural model for the enigmatic long poly-Glu region adjoining the C and D helices of the p28 subunit of IL-27. Thus, we did not model this region of the structure while its structural and functional role remain unclear. Our structure for the mouse IL-27:IL-27Rα complex has used only the CHR segment of IL-27Rα comprising the two N-terminal domains D1 and D2 and lacks the membrane-proximal domains. Furthermore, the reported interaction studies by BLI employing mouse IL-27 and cognate receptors reported in Figure 4 did not employ full length ectodomains of IL-27Rα and gp130. Finally, the surface plasmon resonance experiments reported in Figure S1E as part of efforts to characterize the antagonistic nature of SRF388 binding to human IL-27 with respect to human gp130-Fc or human IL-27Rα-Fc, represent one experiment performed using optimized assay conditions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111490.

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AUTHOR CONTRIBUTIONS

K.S. carried out recombinant protein expression with the help of Y.B. and R.M. M.P. contributed to cell culture and protein production. C.M. contributed to nanobody selection and production. K.S. carried out protein crystallization with contributions from Y.B. and S.N.S. The crystal structure of the mouse IL-27 complex was determined by Y.B. and K.S. with contributions from S.N.S. and F.B. A.S. provided computational support. K.S. designed and performed BLI experiments with the help of Y.B. and M.P. D.A. designed and performed flow cytometry experiments on mouse IL-27 with contributions from C.A.H. J.S., M.W., and D.T.L. contributed to the crystal structure of hIL-27:SRF388 with contributions from Y.B. in crystallographic refinement. K.F.W. performed pSTAT1 experiments and analyzed data with contributions from J.H. and J.A.H. Display items were made by K.S. and Y.B. with input from S.N.S. K.S. and S.N.S. wrote the manuscript with input from all authors. S.N.S. and J.A.H. conceived the project and provided supervision and coordination of research activities.

DECLARATION OF INTERESTS

J.S., K.F.W., and J.A.H are former or current employees and stockholders of Surface Oncology. C.A.H. J.S., M.W., and D.T.L. contributed to the crystal structure of hIL-27:SRF388 with contributions from Y.B. in crystallographic refinement. K.F.W. performed pSTAT1 experiments and analyzed data with contributions from J.H. and J.A.H. Display items were made by K.S. and Y.B. with input from S.N.S. K.S. and S.N.S. wrote the manuscript with input from all authors. S.N.S. and J.A.H. conceived the project and provided supervision and coordination of research activities.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research.

REFERENCES

Andrews, C., McLean, M.H., and Durum, S.K. (2016). Interleukin-27 as a novel therapy for inflammatory bowel disease: a critical review of the literature. Inflamm. Bowel Dis. 22, 2255–2264. https://doi.org/10.1097/MIB.0000000000000815.

Aricescu, A.R., Lu, W., and Jones, E.Y. (2006). A time- and cost-efficient system for high-level protein production in mammalian cells. Acta Crystallogr. D Biol. Crystallogr. 62, 1243–1250. https://doi.org/10.1107/S0907444906029799.

Awasthi, A., Carrier, Y., Peron, J.P.S., Bettelli, E., Kamanaka, M., Flavell, R.A., Kuchroo, V.K., Oukka, M., and Weiner, H.L. (2007). A dominant function for interleukin 27 in generating interleukin-10 producing anti-inflammatory T cells. Nat. Immunol. 8, 1380–1389. https://doi.org/10.1038/ni1541.

Baek, M., DiMaio, F., Anishchenko, I., Dauparas, J., Ovchinnikov, S., Lee, G.R., Wang, J., Cong, Q., Kinch, L.N., Schaeffer, R.D., et al. (2021). Accurate prediction of protein structures and interactions using a three-track neural network. Science 372, 871–876. https://doi.org/10.1126/science.abj8754.

Batten, M., Li, J., Yi, S., Klijavin, N.M., Danilenko, D.M., Lucas, S., Lee, J., de Sauvage, F.J., and Ghirardi, N. (2006). Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat. Immunol. 7, 929–936. https://doi.org/10.1038/nijm.2006.93.
Esch, A., Masiarz, A., Mossner, S., Moll, J.M., Grützinger, J., Schröder, J., Scheier, J., and Floss, D.M. (2020). Deciphering site 3 interactions of interleukin 12 and interleukin 23 with their cognate murine and human receptors. J. Biol. Chem. 295, 10478–10492. https://doi.org/10.1074/jbc.RA210.013935.

Evans, R. (2021). Protein complex prediction with AlphaFold-Multimer. Preprint at bioRxiv. https://doi.org/10.1101/2021.04.06.30034.

Fitzgerald, D.C., Zhang, G.X., El-Behi, M., Fonseca-Kelly, Z., Li, H., Yu, S., Saris, C.J.M., Gran, B., Ciric, B., and Rostami, A. (2007). Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. Nat. Immunol. 8, 1372–1379. https://doi.org/10.1038/nijm.

Floss, D.M., Moll, J.M., and Scheier, J. (2020). IL-12 and IL-23-closse relatives with structural homologies but distinct immunological functions. Cells 9, 2184. https://doi.org/10.3390/cells9102184.

Georgy, J., Artt, Y., Moll, J.M., Ouzin, M., Weitz, H.T., Gremer, L., Willbold, D., Grützinger, J., Thves-Kurenbach, F., Scheier, J., and Floss, D.M. (2021). Trystophan (W) at position 37 of murine IL-12/IL-23 p40 is mandatory for binding. J. Biol. Chem. 297, 101295. https://doi.org/10.1016/j.jbc.2021.101295.

Hasegawa, H., Mizoguchi, I., Chiba, Y., Ohashi, M., Xu, M., and Yoshimoto, T. (2016). Expanding diversity in molecular structures and functions of the IL-6/IL-12 heterodimeric cytokine family. Front. Immunol. https://doi.org/10.3389/fimmu.2016.00479.

Groätzinger, J., Thives-Kurenbach, F., Scheller, J., and Floss, D.M. (2021). Structural basis for IL-12 and IL-23 receptor sharing reveals a gateway for shaping actions on T versus NK cells. Cell 184, 983–999.e24. https://doi.org/10.1016/j.cell.2021.01.018.

Hasegawa, H., Mizoguchi, I., Chiba, Y., Ohashi, M., Xu, M., and Yoshimoto, T. (2016). Expanding diversity in molecular structures and functions of the IL-6/IL-12 heterodimeric cytokine family. Front. Immunol. 7, 479. https://doi.org/10.3389/fimmu.2016.00479.

Hildenbrand, I., Aschenbrenner, I., Franke, F.C., Devergne, O., and Feige, M.J. (2019b). A folding switch regulates interleukin 27 biogenesis and secretion of its alpha-subunit as a cytokine. Proc. Natl. Acad. Sci. USA 116, 1585–1590. https://doi.org/10.1073/pnas.1816698116.

Nguyen, C.T., Bloch, Y., Skladanowska, K., Savvides, S.N., and Adamopoulos, I.E. (2019). Pathophysiology and inhibition of IL-23 signaling in psoriatic arthritis: a molecular insight. Clin. Immunol. 206, 15–22. https://doi.org/10.1016/j.clim.2018.09.002.

Patnaik, A., Morgenstern, D., Mantia, C., Tannir, N.M., Harshman, L.C., Hill, J., White, K., Chung, J.-K., Bowers, B., Sciaranghella, G., et al. (2021). Results of a phase 1 study of SRF388, a first-in-human, first-in-class, high-affinity anti-IL-27 antibody in advanced solid tumors. J. Clin. Oncol. 39, 2551. https://doi.org/10.1002/jco.2021.09.15.suppl.2551.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Eng, E.C., Couch, G.S., Croll, T.I., Morris, J.H., and Ferrin, T.E. (2021). UCSF ChimeraX: Structure visualization for researchers, educators, and developers. Protein Sci 30, 70–82. https://doi.org/10.1002/pro.3943.

Pflanz, S., Hibbert, L., Mattson, J., Rosales, R., Vaisberg, E., Bazan, J.F., Phillips, J.H., McClanahan, T.K., de Waal Malefyt, R., and Kastelein, R.A. (2004). WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. J. Immunol. 172, 2225–2231. https://doi.org/10.4049/jimmunol.172.4.2225.

Pflanz, S., Timans, J.C., Cheung, J., Rosales, R., Kanzer, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., et al. (2002). IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells. Immunity 16, 779–790. https://doi.org/10.1016/s1074-7613(02)00324-2.

Pylayeva-Gupta, Y. (2016). Molecular pathways: interleukin-35 in autoimmunity and cancer. Clin. Cancer Res. 22, 4973–4978. https://doi.org/10.1158/1078-0432.CCR-16-0743.

Ramanurthy, V., Krystek, S.R., Jr., Bush, A., Wei, A., Emanuel, S.L., Das Gupta, R., Janus, A., Cheng, L., Murdock, M., Abramczyk, B., et al. (2012). Structures of adenin/protin complexes reveal an expanded binding footprint. Structure 20, 259–269. https://doi.org/10.1016/j.str.2011.11.016.

Reeves, P.J., Callevaert, N., Contreras, R., and Khorana, H.G. (2002). Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acytelyglucosaminyltransferase I-negative HEK293S stable mammalian cell line. Proc. Natl. Acad. Sci. USA 99, 13419–13424. https://doi.org/10.1073/pnas.121519299.

Rousseau, D., Basset, L., Froger, J., Dinguirard, N., Chevalier, S., and Gascan, H. (2010). IL-27 structural analysis demonstrates similarities with ciliary neurotrophic factor (CNTF) and leads to the identification of antagonistic variants. J. Mol. Biol. 402, 797–812. https://doi.org/10.1016/j.jmb.2010.07.046.

Min, B., Kim, D., and Feige, M.J. (2021). IL-30(dagger) (IL-27A): a familiar stranger in immunity, inflammation, and cancer. Exp. Mol. Med. 53, 823–834. https://doi.org/10.1038/s12276-021-00630-x.

Mirdita, M., Schütze, K., Moriwicki, Y., Heo, L., Ovchinnikov, S., and Steinberger, M. (2022). ColabFold - making protein folding accessible to all. Preprint at bioRxiv. https://doi.org/10.1101/2021.08.15.454625.

Müller, S.I., Aschenbrenner, I., Zacharias, M., and Feige, M.J. (2019a). An interspecies analysis reveals molecular construction principles of interleukin 27. J. Mol. Biol. 431, 2383–2393. https://doi.org/10.1016/j.jmb.2019.04.032.

Müller, S.I., Friedl, A., Aschenbrenner, I., Esser-von Bieren, J., Zacharias, M., Deverge, O., and Feige, M.J. (2019b). A folding switch regulates interleukin 27 biogenesis and secretion of its alpha-subunit as a cytokine. Proc. Natl. Acad. Sci. USA 116, 1585–1590. https://doi.org/10.1073/pnas.1816698116.
Subedi, G.P., Johnson, R.W., Moniz, H.A., Moremen, K.W., and Barb, A. (2015). High yield expression of recombinant human proteins with the transient transfection of HEK293 cells in suspension. JoVE, e53568. https://doi.org/10.3791/53568.

Terwilliger, T.C., Poon, B.K., Afonine, P.V., Schlicksup, C.J., Croll, T.I., Millán, C., Richardson, J.S., Read, R.J., and Adams, P.D. (2022). Improved AlphaFold modeling with implicit experimental information. Preprint at bioRxiv. https://doi.org/10.1101/2022.01.07.475350.

Tormo, A.J., Beaupré, L.A., Elson, G., Crabé, S., and Gauchat, J.F. (2013). A polyglutamic acid motif confers IL-27 hydroxyapatite and bone-binding properties. J. Immunol. 190, 2931–2937. https://doi.org/10.4049/jimmunol.1201460.

Vagin, A., and Teplyakov, A. (1997). MOLREP: an automated program for molecular replacement. J. Appl. Crystallogr. 30, 1022–1025. https://doi.org/10.1107/S0021889897006766.

Verstraete, K., Peelman, F., Braun, H., Lopez, J., Van Rompaey, D., Dansercoer, A., Vandenberghe, I., Pauwels, K., Tavernier, J., Lambrecht, B.N., et al. (2017). Structure and antagonism of the receptor complex mediated by human TSLP in allergy and asthma. Nat. Commun. 8, 14937. https://doi.org/10.1038/ncomms14937.

Verstraete, K., van Schie, L., Vyncke, L., Bloch, Y., Tavernier, J., Pauwels, E., Peelman, F., and Savvides, S.N. (2014). Structural basis of the proinflammatory signaling complex mediated by TSLP. Nat. Struct. Mol. Biol. 21, 375–382. https://doi.org/10.1038/nsmb.2794.

Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T., and Bricogne, G. (2011). Data processing and analysis with the autoPROC toolbox. Acta Crystallogr. D Biol. Crystallogr. 67, 293–302. https://doi.org/10.1107/S090744811007773.

Tait Wojno, E.D., Hunter, C.A., and Stumhofer, J.S. (2019). The immunobiology of the interleukin-12 family: room for discovery. Immunity 50, 851–870. https://doi.org/10.1016/j.immuni.2019.03.011.

Yoshida, H., and Hunter, C.A. (2015). The immunobiology of interleukin-27. Annu. Rev. Immunol. 33, 417–443. https://doi.org/10.1146/annurev-immunol-032414-112134.

Zhang, H., Madi, A., Yosef, N., Chihara, N., Awasthi, A., Pot, C., Lambda, C., Srivastava, A., Burkett, P.R., Nyman, J., et al. (2020). An IL-27-driven transcriptional network identifies regulators of IL-10 expression across T helper cell Subsets. Cell Rep. 33, 108433. https://doi.org/10.1016/j.celrep.2020.108433.
# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| TCRb ε450           | Invitrogen | Cat#48-5961-82; RRID: AB_11039532 |
| CD11b BV605         | BioLegend | Cat# 101257; RRID: AB_2565431 |
| CD19 BV650          | BioLegend | Cat# 115541; RRID: AB_11204087 |
| NK1.1 BV711         | BioLegend | Cat# 108745; RRID: AB_2563286 |
| CD90.2 AF700        | BioLegend | Cat# 105319; RRID: AB_493724 |
| CD4 APC-e780        | Invitrogen | Cat# 47-0041-82; RRID: AB_11218896 |
| pSTAT1 (pY701) PE | BD Biosciences | Cat# 562069; RRID: AB_11151907 |
| pSTAT3 (pY705) AF647 | BD Biosciences | Cat# 557815; RRID: AB_647144 |
| pSTAT3 (pY705) PE | BD Biosciences | Cat#612569 |
| Human gp130 Ab, mouse IgG1 | R&D Systems | Cat#MAB628-500 Clone#28105 |
| Human gp130 Ab, mouse IgG1 | R&D Systems | Cat#MAB228-500 Clone#28126 |
| rhgp130 Fc Chimera, human IgG1, | R&D Systems | Cat#671-GP-100 |
| Anti-CD130 (gp130) antibody B-T2 | Abcam | Cat#ab11462 |
| Mouse IL-27 p28/IL-30 monoclonal antibody | R&D Systems | Cat# MAB7430 |
| EB13 Polyclonal Antibody | Thermo Fisher Scientific | Cat# PA5-23297 |
| Mouse IL-27r alpha/WSX-1/TCCR Antibody | R&D Systems | Cat# MAB21091-SP |
| IRDye 680RD Goat Anti-Rat IgG Secondary Antibody | Westburg | Cat# 926-68076 |
| IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody | Westburg | Cat# 926-32211 |
| Brilliant Violet 421 (BV421) mouse anti-rat CD3 (BV421-CD3) | BD Biosciences | Cat#563948 |
| Fluorescein isothiocyanate (FITC) mouse anti-human CD3e (FITC-CD3e) | BD Biosciences | Cat#556611 |
| FITC mouse anti-human CD3 (FITC-CD3); | BD Biosciences | Cat#561807 |
| Fluorescein isothiocyanate (FITC) rat anti mouse CD3 (FITC-CD3) | BD Biosciences | Cat#555274 |
| Bacterial and virus strains |        |            |
| E. coli BL21 T7 Express lys/Iq | New England Biolabs | Cat #C3013I |
| Biological samples |        |            |
| Rat whole blood | Bioreclamation IVT (Westbury, NY) | | |
| Cynomolgus monkey whole blood | Bioreclamation IVT (Westbury, NY) | | |
| Mouse whole blood from 4-10 week old female Balb/c mice | Taconic | | |
| Human blood from normal healthy volunteers | Research Blood Components (Boston, MA) | | |
| Frozen PBMC pool (KP51371 KP51373 KP51374) | Research Blood Components, LLC | | |
| Chemicals, peptides, and recombinant proteins |        |            |
| mp28, mEBI3, mIL-27α, mIL-27β, mgp130CHR, mgp130CHR NAKS | This paper, plasmids are available at BCCM/Gene Corner Plasmid Collection, Ghent University; https://www.genecorner.ugent.be | Cat# LMBP 13424, LMBP 13425, LMBP 13426, LMBP 13423, LMBP 13422, LMBP 13421, LMBP 13427 |
| Phosphatase Inhibitor Cocktail | Sigma-Aldrich | Cat#P5726 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant murine IL-27 | BioLegend | Cat# 577402 |
| Recombinant human heterodimeric IL-27 (BLI) | PeproTech | Cat#200-38 |
| Recombinant cynomolgus monkey IL-27 (BLI) | R&D Systems | Cat#CUST01701 |
| Recombinant rat IL-27 (BLI) | WuXi Biologics | Cat#784-S54 |
| Recombinant mouse IL-27 (BLI) | R&D Systems | Cat# 7430-ML-010/CF |
| Recombinant mouse IL-27 (STAT inhib.) | R&D Systems | Cat# 2799-ML |
| Recombinant human IL-27 (linked - STAT inhib) | R&D Systems | Cat# 2526-IL-010 |
| SRF388 Fab antibody | Patent | US20190382474A1 |
| hlIL-27Rα-Fc biotin | WuXi Biologics | Lot: 2016-08-26 |
| hgp130-Fc-biotin | WuXi Biologics | Lot: 2016-08-26 |
| rhIL-27 | R&D Systems | Cat#2526-IL-010 |
| rhIL-6 | eBioscience | Cat# 14-8069-62 |

Critical commercial assays

| Octet® Streptavidin (SA) Biosensor | Sartorius | Cat# 18-5020 |
| Octet® Anti-HlgG Fc Capture (AHC) Biosensors | Sartorius | Cat# 18-5060 |
| ProteOn™ GLM sensor chip | Bio-Rad | Cat# 176-5012 |

Deposited data

| IL-27:IL-27Rα:Nb5 | This paper | PDB: 7zg0 |
| IL-27:SRF388Fab | This paper | PDB: 7zxk |

Experimental models: Cell lines

| Human: HEK293S | (Lin et al., 2014) |
| Human: HEK293 S MGAT1−/− | (Reeves et al., 2002) |
| U937 | ATCC #CRL-1593.2 |

Recombinant DNA

| pHLSec parental vector | (Aricescu et al., 2006) |
| pMECS parental vector | VIB Nanobody Service Facility | https://corefacilities.vib.be/nsf |

Software and algorithms

| Astra 6.1 | Wyatt |
| Alphafold | (Evans, 2021; Jumper et al., 2021; Mirdita et al., 2022; Terwilliger et al., 2022). |
| Buster | (Bricogne et al., 2017) |
| ChimeraX | (Pettersen et al., 2021) |
| Coot | (Emsley et al., 2010) |
| CrystalsPro version 41.119a | Rigaku |
| GraphPad Prism Software version 9 | GraphPad |
| Molrep | (Vagin and Teplyakov, 1997) |
| Octet® Analysis Studio 12.2.1.24 | Sartorius |
| Phenix | (Liebschner et al., 2019) |
| Pymol | Schrödinger, LLC |
| XDS | (Kabsch, 2010) |

Other

| Mini Protean TGX Precast Gel Any kD | Bio-Rad | Cat# 456-9035 |
| Precision Plus Protein Dual Color Standard | Bio-Rad | Cat# 161-0374 |
| JCSG+ | Molecular Dimensions | Cat# MD1-37 |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Savvas Savvides (savvas.savvides@ugent.be).

Materials availability
The HEK293 S MGAT1−/− cell line and derivatives thereof cannot be freely distributed as some rights remain with the original authors.

Data and code availability

- Coordinates and structure factors for the crystal structures of IL-27:IL-27Rα:Nb5 complex and human IL-27:SRF388Fab have been deposited to the Protein Data Bank (http://www.rcsb.org/) with accession codes 7zg0 and 7zxk, respectively.
- The “Disentangle” Perl script to correct for overlapping reflections arising from the non-merohedral diffraction pattern is available online at https://github.com/asoete/disentangle and https://doi.org/10.5281/zenodo.7050958.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
Suspension-adapted HEK293 S cells and HEK293 S MGAT1−/− cells, which lack Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase enzyme are derived from HEK293 cells which are female and were grown serum-free in a 1:1 mixture of Freestyle (ThermoFisher) and Ex-Cell (Sigma-Aldrich) medium at 37°C, 75% humidity, CO2 at 8% and shaking speed of 130 rpm (with shaker’s orbital eccentricity at 25 mm). HEK293 S MGAT1−/− cells were provided by Prof. N. Callewaert, Unit for Medical Biotechnology, VIB-UGent Center for Medical Biotechnology, Ghent, Belgium. The HEK293 S MGAT1−/− cell line and derivatives thereof cannot be freely distributed as some rights remain with the original authors.

METHOD DETAILS

Plasmids and cell lines for protein expression in mammalian cells
For protein production in HEK293 cells all constructs were cloned into the pHLsec plasmid in frame with an N-terminal chicken RTPμ-like secretion signal (Aricescu et al., 2006) with the exception of mIL-27sc, which contains native secretion signal of EBI3. Sequence-optimized cDNA encoding the reference sequences of mp28 NM_145636.1 (residues Met1-Ser243) and mEBI3 NM_015766.2 (residues Met1-Pro228) were ordered from IDT. mp28 res Phe29-Ser234 were cloned via Agel and KpnI sites in frame with a non-cleavable C-terminal hexahistidine tag, mEBI3 residues Tyr19-Pro228 were cloned via Agel and XhoI sites without any tags. mIL-27sc was generated via overlap extension PCR by inserting a (GGGS)4 linker between mEBI3 residues Mey1-Pro228 and mp28 Phe29-Ser234 and cloned into the pHLsec backbone in frame with a C-terminal caspase-3 site followed by an Avi-His tag. Sequence-optimized cDNA encoding mIL-27Rα residues Met1-Lys510 (NM_016671.3) and mpg130 residues Met1-Glu617 (NM_010560.3) were purchased from Genscript. Sequences corresponding to the mIL-27RαCHR (residues Thr25-Leu225) and mpg130CHR (residues Glu23-Arg323) were cloned in frame with an N-terminal octa-histidine tag followed by a TEV cleavage site using Agel and Xhol restriction sites. Mouse gp130CHR (residues Phe124-Arg323) was cloned in frame with an N-terminal His-MBP tag followed by a TEV cleavage site.

Production of recombinant proteins for X-ray crystallography
Protein production for crystallographic purposes was performed in suspension-adapted HEK 293 MGAT1−/− cells, which were obtained from prof. N. Callewaert, Unit for Medical Biotechnology, VIB-UGent Center for Medical Biotechnology, Ghent, Belgium. Cells were grown serum-free in a 1:1 mixture of Freestyle (ThermoFisher) and Ex-Cell (Sigma-Aldrich) medium according to the protocol (Subedi et al., 2015) with modifications. Transient transfection was performed in Freestyle medium with linear polyethylenimine 25 kDa (Polysciences) with DNA to PEI ratio of 1:2. Cells were centrifuged at 500 RCF for 5 min and resuspended in pure Freestyle.
Production of recombinant proteins for bio-layer interferometry (BLI)

Protein production for the BLI experiments was performed in suspension-adapted HEK293 S with native glycosylation. The cell line was obtained from prof. N. Callewaert, Unit for Medical Biotechnology, Medical Biotechnology Center, VIB-UGent, Ghent, Belgium. Plasmids containing mIL-27R\textsubscript{CHR}, mp130\textsubscript{CHR}, mp130\textsubscript{IgCHR} or mpg130\textsubscript{CHR} His-MBP were transiently transfected as described above. mIL-27\textsubscript{sc} Avi-His was transiently co-transfected with a BirA expression plasmid (pDisplayBirA-ER45) in 4:1 ratio (Howarth and Ting, 2008). Expression medium was supplemented with 100 \mu M biotin upon transfection. Six days post-transfection conditions medium was collected and purified using the 1 mL cOmplete\textsuperscript{TM} His-Tag purification column (Roche) and further cleaned-up via SEC and size exclusion chromatography (SEC) using the Superdex 200 increase 10/300 GL (Cytiva). Nb5 obtained from \textit{E. coli} was added to the IL-27:IL-27R\textsubscript{x} complex in a 1:2 molar ratio. Glycans were trimmed by overnight enzymatic digestion at 4°C with EndoglycosidaseH (house made) in 1:100 ratio. Simultaneously the His-tag on the Nb5 was removed by caspase-3 digestion. Caspase-3 was also made in-house and was used in 1:100 ratio. On the following day IL-27:IL-27R\textsubscript{x}:Nb5 complex was further purified using Superdex 200 increase 10/300 GL column to remove excess Nb5 and enzymes. Protein was concentrated to 4.5 mg/mL and crystallization screens were set-up.

Bio-layer interferometry of mouse IL-27 binding to cognate receptors

The binding kinetics and dissociation constant of IL-27 toward its receptors: murine IL-27R\textsubscript{CHR} and mpg130\textsubscript{IgCHR} were characterized by bio-layer interferometry (BLI). Binding assays were performed using an Octet Red 96 (Forte' BIO) in assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (w/v) BSA, 0.02% (v/v) Tween 20) at 25°C. Biotinylated mIL-27\textsubscript{sc} Avi-His was immobilized at 1.25 \mu g/mL in HBS onto Octet\textsuperscript{TM} SA capture biosensors (Sartorius) until an optical shift of 1.5 nm was achieved. The sensors were subsequently quenched with a 10 \mu g/mL biotin solution, equilibrated in assay buffer and then exposed to a 2-fold dilution series of the ligand (a) IL-27R\textsubscript{CHR}: 3.13–100 nM, (b) mpg130\textsubscript{IgCHR}: 6.25–200 nM, (c) mpg130\textsubscript{CHR}: 6.25–200 nM in the presence of a constant saturating concentration of the mIL-27R\textsubscript{x} (100 nM), (d) Nb5: 0.625–20 nM or (e) IL-27R\textsubscript{CHR}: 3.13–100 nM in the presence of a constant saturating concentration of the Nb5 (25 nM). To verify that no non-specific binding was present during the interaction assay, non-functionalized biosensor was used as a control by measuring the highest ligand concentration. All biosensors were discarded after each cycle. The sensor traces from zero concentration samples were subtracted from the raw data traces before data analysis. After subtraction of the control sensorgrams, a 1:1 binding model was fitted to each dilution series. Assay design and data acquisition were performed using the Octet\textsuperscript{R} BLI Discovery data acquisition software 12.2.1.18 (Sartorius) and data analysis was performed using the Octet\textsuperscript{R} Analysis Studio 12.2.1.24 (Sartorius). All binding experiments were performed in triplicate. Displayed \( K_D \), \( k_a \) and \( k_s \) values represent the averages of these triplicates.

Bio-layer interferometry (BLI) of human, mouse, monkey and rat IL-27 binding to SRF388

The hIL-27 was composed of 2 subunits (p28 and EB13) purified as a heterodimer. The recombinant mouse, rat, and cynomolgus monkey IL-27 was expressed and purified as a linked heterodimer with p28 and EB13 subunits tethered together by a glycine-serine linker. This analysis utilized anti-human IgG Fc capture (AHC) biosensors (Sartorius) pre-loaded with anti-human Fc antibody to first bind SRF388 (ligand) in assay buffer (phosphate buffered saline, 0.1% BSA, 0.02% Tween 20, 0.05% sodium azide, pH 7.4) in a 96-well plate format to measure association and dissociation of IL-27 (analyte) at 7 concentrations (1:1 serial dilution beginning at 5 \mu g/mL for human and monkey IL-27 and 2.5 \mu g/mL for mouse and rat IL-27) to the SRF388 antibody using an Octet Qke (ForteBio). One biosensor was utilized as a reference channel for background subtraction. The experiments for human and monkey IL-27 binding were performed with 1.0 \mu g/mL SRF388 loaded onto the AHC biosensor. The experiments for rat and mouse species cross-reactivity were conducted in a similar manner with a lower loading concentration of the SRF388 ligand onto the biosensor (0.75 \mu g/mL vs 1.0 \mu g/mL). A shorter association time for hIL-27 was required for the experimental conditions due to the very strong interaction this protein has with SRF388. Similarly, the longer dissociation time for the human protein was needed to better define the off-rate and the fit of the 1:1 binding model with SRF388. The ultra-high affinity measured by BLI was confirmed utilizing MSD.
solution-phase equilibrium K_d measurement testing biotinylated GGS hIL-27 and SRF388 Fab (3.4 pM, data not shown). Monkey, rat, and mouse IL-27 had lower affinities than hIL-27 and were therefore run with longer association and shorter dissociation times. BLI binding data was analyzed with Octet Data Analysis software v10.0.1.7 (ForteBio).

**Production of single domain VHH camelid antibody against mL-27**
Single domain camelid antibodies (nanobodies) were raised against murine IL-27:IL-27R_αCHR-gp30_βCHR complex by immunizing a llama with IL-27:IL-27R_αCHR-gp30_βCHR complex and were selected for specific binding to this complex via several rounds of panning in the Nanobody Service Facility, VUB, Brussels. However, additional characterization by ELISA revealed that all the binders were specific towards IL-27 alone, thus they could be used to search for a crystallographic adjuvant for the binary complex as well. Several candidates were expressed and their off-rates characterized via BLI using crude lysates (data not shown). Six best Nbs were expressed and purified in *E. coli* and used in crystallization trials with the binary complex. Nb5 was the VHH which produced crystals in complex with IL-27:IL-27R_αCHR.

**Production of an anti-mIL-27 single domain VHH camelid antibody in *E. coli***
The MCS of the pET15b plasmid including the NcoI site were replaced by a Met and pelB secretion signal followed in frame by an N-terminal hexahistidine tag, a caspase 3 cleavage site (Asp-Glu-Val-Asp), and an AgeI site. Finally, sequence encoding Nb5 was pended in 200 μg/mL to a final concentration of 2% + 1:100 phosphatase. Cells were fixed for 20 min, spun down, and resus-

**Phosphorylation of STAT1/3 and pSTAT1/3 flow cytometry analysis**
Single cell suspensions were prepared by passing spleens through a 70 μm strainer and washing with RPMI supplemented with 5% FBS. Red blood cells were then lysed by incubating samples with ACK lysis buffer (Thermo Fisher Scientific) for 5 min before washing again with RPMI +5% FBS. 4 × 10^6 cells/stimulation condition were then plated in a 96 well U-bottom plate. Cells were washed with FACS Buffer [1× PBS, 0.02% bovine serum antigen, 1 mM EDTA] before incubating with Fc block (99.5% FACS Buffer, 0.5% normal rat serum, 1 μg/mL 2,4-DG IgG antibody) prior to staining. Cells were stained with the viability dye Ghost Dye Violet 510 (Tonbo biosciences; 12–0870), and the following antibodies were used for subsequent staining; TCRb ef450 (clone: H57-597), CD11b BV605 (clone: M1/70), CD19 BV650 (clone: 6D5), NK1.1 BV711 (clone: PK136), CD4 APC-ef780 (clone: GK1.5). Cells were then stimulated with either 0, 5, 10, 20, or 40 ng/mL of prewarmed (37°C) mL-27, mL-27sc or mL-27scBL. Cells were incubated at 37°C for 30 min to induce phosphorylation of STAT1/3 before adding 4% PFA+1:50 phosphatase inhibitor cocktail (Sigma-Aldrich) solution to a final concentration of 2% + 1:100 phosphatase. Cells were fixed for 20 min, spun down, and resus-

**Inhibition of STAT1 phosphorylation in T cells from mouse, rat, cynomolagus monkey, and human whole blood by SRF388**
Rat, cynomolagus monkey, and human blood was purchased from commercial vendors. Murine blood was pooled from Balb/c mice (n = 10/test). Monkey, mouse and human IL-27 were purchased from commercial vendors and til IL-27 was produced by WuXi Biologics. All recombinant forms of IL-27 were GGS linked versions of the cytokine. Blood samples were added to 96-well plates and treated in duplicate with SRF388 or IgG1 isotype control antibody at concentrations ranging from 20 μg/mL to 1 ng/mL for 1 h. Species-specific recombinant IL-27 at 18.2 ng/mL was added to the antibody-treated and stimulated control wells, which represented 0% inhibition. Phosphate buffered saline (PBS) was added to unstimulated control wells, which represented 100% inhibition. The plates were incubated for 30 min. Red blood cells were lysed, white blood cells were fixed, and cells were incubated with a species-specific fluorescently conjugated CD3 antibody to label T cells. For cynomolagus monkey samples a cross-reactive mouse anti-human antibody (FITC-CD3e) was used. Cells were permeabilized with Perm Buffer III, incubated with phycoerythrin mouse anti-STAT1 (pY701) (PE-pSTAT1) antibody, and analyzed by flow cytometry. The geometric mean fluorescence intensity (gMFI) of PE-pSTAT1 in CD3+ T cells was determined and percent inhibition was calculated in FlowJo_V10 Software (Ashland, Oregon). Raw data were normalized to the stimulated control level (100% inhibition) and unstimulated control level (0% inhibition). Percent inhibition values were plotted in GraphPad Prism software and IC50 was determined using a 4-parameter nonlinear regression equation.

**Inhibition of STAT1 or STAT3 phosphorylation in U937 cells and human T cells from PBMCs by anti-gp130 antibodies**
U937 cells or ficoll-isolated human PBMCs were cultured in RPMI with various concentrations of anti-gp130 antibodies and either rhIL-27 or rhIL-6 (20 ng/mL) for 20 min at 37°C. Cells were then fixed by the addition of 4% PFA for 5 min followed by washing in
DPBS. Cells were incubated with ice-cold 90% MeOH (in deionized water) at −20°C for 15 min followed by washing and staining for pSTAT1 (pY701) or pSTAT3 (pY705). Samples were washed with FACS buffer, read on a LSR Fortessa (BD Biosciences), and analyzed using the FlowJo Software analysis program (TreeStar). Cytokine stimulated conditions represents 0% inhibition and unstimulated conditions represents 100% inhibition.

**hIL-27Rx and hgp130 blocking studies via surface plasmon resonance**

Surface plasmon resonance (SPR) experiments were performed using the ProteOn™ XPR36 Protein Interaction Array System according to the user manual. Activated ProteOn GLM sensor chips were used to couple hIL-27Rx-Fc or hgp130-Fc onto the streptavidin surface via the biotin tag. Chips were rotated 90° and washed with running buffer until the baseline was stable. Mixtures of hIL-27 and SRF388 Fab were prepared in 1:10 molar ratio and incubated 2 h at 25°C. The solution was injected at a flow rate of 50 μL/min for an association phase of 200 s, followed by 2000 s dissociation for the chip loaded with hgp130-Fc or for an association phase of 120 s, followed by 60 s dissociation for chip loaded with hIL-27Rx-Fc. Chips were regenerated by injecting 10 mM glycine pH 1.5 following every dissociation phase. Subsequently hIL-27 without the addition of SRF388 was also injected. Sensograms for the reference channel containing no analyte were subtracted from the test sample (both hIL-27Rx-Fc and hgp130-Fc with rhIL-27 or rhIL-27 + SRF388). Molecular weights of 60 kDa and 150 kDa were used to calculate the molar concentration of IL-27 analyte following every dissociation phase. Subsequently hIL-27 without the addition of SRF388 was also injected. Sensograms for the reference channel containing no analyte were subtracted from the test sample (both hIL-27Rx-Fc and hgp130-Fc with rhIL-27 or rhIL-27 + SRF388). Molecular weights of 60 kDa and 150 kDa were used to calculate the molar concentration of IL-27 analyte following every dissociation phase.

**mIL-27:mIL-27Rx:Nb5 crystal structure determination and refinement**

Commercial sparse matrix sitting drop crystallization screens were set up using the Mosquito liquid handling robot (TTP Labtech) using a 100 nL of IL-27:IL-27R complex at 4.5 mg/mL mixed with 100 nL mother liquor in SwissSci 96-well triple drop plates. Plates were incubated at 14°C. Crystals initially appeared after 18 days in Proplex G9 (1.3 M sodium/potassium phosphate, pH 7.0) and Proplex G10 (1.6 M sodium/potassium phosphate, pH 6.5) condition of the screen (Molecular Dimensions), however multiple optimization efforts failed to produce crystals of acceptable diffraction quality. Microseed matrix screening (MMS) (D’Arcy et al., 2007) into various sparse matrix crystallization screens was implemented and yielded crystals in multiple new conditions. Seed stock was prepared with the Seed Bead PTFE Kit (HR-320, Hampton Research) according to the manufacturer’s instructions. IL-27:IL-27Rx:Nb5 complex at 4.2 mg/mL was mixed with the seed stock in the 2:1 ratio and set up in sitting drop format as described above. Initial hit from Wizard H12 (Molecular Dimensions) (1 M Potassium sodium tartrate, 0.1 M MES/Sodium hydroxide pH 6.0) was further seeded into a custom Wizard H12 optimization screen. The best diffracting crystals were obtained by seeding with a 1:10 dilution of the new seed stock obtained from the original Wizard H12 hit. Sitting drop plates were set up using the Mosquito robot with 400 nL of protein concentrated to 5.2 mg/mL mixed with 200 nL of mother liquor (1 M Potassium sodium tartrate, 0.1 M MES/Sodium hydroxide pH 5.9) and incubated at 20°C. Crystals were cryoprotected with 4 M sodium formate solution mixed in 3:1 ratio with mother liquor prior to being cryo-cooled in liquid nitrogen. Diffraction data was collected at 100 K at the P14 microfocus beam line at PETRA III, Hamburg, Germany. The data was integrated using XDS (Kabsch, 2010). The data was found to be somewhat pathological in nature. For all of the crystals that yielded complete datasets, only about half of the diffraction spots could be indexed and attributed to the C2 lattice at any given time. The other half of the spots indexed to a near identical C2 lattice, which was oriented rotated 180° along b from the first one. The relationship between both lattices is however non-merohedral and as such there is no twin law applicable that can correct for this pathology. The off-center peak in the Patterson map would coincide with such an interpretation of the lattices. To correct for overlapping reflections arising from the non-merohedral diffraction pattern we wrote a “disentangle” Perl script that takes as input the INTEGRATE.HKL files integrated from each of the lattices (https://strucbio.biologie.uni-konstanz.de/xdswiki/index.php/Indexing-Indexing_images_from_non-merohedrally_twinned_crystals_28i.e_.several_lattices.29) and calculates the pairs-wise distances on the diffraction images of the spots between both lattices. The distance in z is assumed to be 1 pixel per subsequent frame in the precession, which is a simplification. Spots belonging to a lattice for which another spot was found nearby belonging to the other lattice are then excluded. A resulting INTEGRATE.HKL file containing only non-overlapping spots of either lattice is finally written out and can be used as input for the CORRECT step in XDS. The script is available online at https://github.com/asoe/disentangle. Model building an refinement was performed against this corrected data. Final cross validation was also performed against the uncorrected data. Initial phases were obtained using maximum likelihood molecular replacement in Phaser (McCoy et al., 2007) using models of mp28 and mEBI3 obtained from the RoseTTA fold server (Baek et al., 2021). Structure refinement steps were iteratively carried out in Phenix.refine (Liebschner et al., 2019) using individual B-factor refinement in combination with 1 TLS group per domain, followed by manual building in Coot (Emsley et al., 2010). Automatically determined Non-Crystallographic Symmetry (NCS) restraints were applied during initial refinement steps. Prosmart restraints as implemented in Coot iteratively calculated from Alphafold models seeded with the refined structure, allowed for qualitative restrained real-space refinement in Coot, greatly improved model geometry (Jumper et al., 2021; Mirdita et al., 2022; Terwilliger et al., 2022).

**hIL-27:SRF388Fab crystal structure determination and refinement**

Recombinant human heterodimeric IL-27 was purchased from PeproTech. SRF388Fab was transiently expressed in CHO-EBNA cells in shake flask and included the variable domain (VL) and constant domain (CH1) of the SRF388 heavy chain and the variable (VL) and constant (CH1) domains of the IL-27Rα heavy chain. Cells were incubated with ice-cold 90% MeOH (in deionized water) at −20°C for 15 min followed by washing and staining for pSTAT1 (pY701) or pSTAT3 (pY705). Samples were washed with FACS buffer, read on a LSR Fortessa (BD Biosciences), and analyzed using the FlowJo Software analysis program (TreeStar). Cytokine stimulated conditions represents 0% inhibition and unstimulated conditions represents 100% inhibition.
constant (CL) domains of the SRF388 light chain. The clarified supernatant from the shake flask expression was affinity purified to >95% monomer by SE-HPLC and buffer exchanged into 20 mM histidine, 5% (w/w) sucrose, pH 6.5 at 1.34 mg/mL.

The hIL-27 was resuspended in 25 mM Tris-HCl, 250 mM NaCl, pH 7.5 at ~1 mg/mL. An excess amount of SRF388Fab was complexed with the resuspended hIL-27 at ambient temperature. The resulting complex was then purified from the excess SRF388Fab using an SRT-10 (Sepax Technologies) size-exclusion chromatography column (10 µm particle size, 300 Å pore size) on an AKTApure 25 (Cytiva) FPLC using 25 mM Tris-HCl, 250 mM NaCl, pH 7.5 as the mobile phase. The purified complex was then buffer-exchanged by centrifugal ultrafiltration to reduce NaCl and concentrate to ~10 mg/mL. hIL-27 - SRF388Fab complex at 10.1 mg/mL in 25 mM Tris pH 7.5, ~30 mM sodium chloride and 5% glycerol was used to set up crystallization trials.

Crystals were obtained in multiple conditions. The best diffracting crystals were obtained using the PACT screen, condition A2 (0.1 M SPG buffer (succinic acid, sodium dihydrogen phosphate and glycine) pH 5 and 25% PEG 1500). Seed stock was made from these crystals and an MMS experiment using JCSG+ screen was set up. Drops consisted of 200 nL protein +50 nL seed stock +150 nL reservoir and were dispensed into an MRC 3-well plate using a mosquito robot (STP Labtech). Crystals appeared in condition A3 (0.2 M di-ammonium hydrogen citrate and 20% PEG 3350) of the JCSG+ screen grown at 20°C. Crystals were cryo-protected with cryo solution containing 0.2 M di-ammonium hydrogen citrate, 20% PEG 3350 and 25% glycerol prior to being cryo-cooled in liquid nitrogen. Diffraction data was collected at 100 K at station I04, Diamond Light Source, Didcot, UK equipped with an Eiger2 XE 16 M detector. The data were processed using the autoPROC (Vonrhein et al., 2011) pipeline and anisotropically truncated using the STARANISO software also including the Aimless program. Final model validation was also performed against the uncorrected data. The structure was determined using the molecular replacement software Phaser (McCoy et al., 2007) and Molrep (Vagin and Teplyakov, 1997). The search models used for the heavy chain, light chain, p28 and EBI3 were the following PDB files: 6azm, 5ijk, an in-house model of p19 from IL-23 and 5fuc respectively. Two complexes were found in the asymmetric unit, with a solvent content of 55.6%. The structure was iteratively refined using Buster (Bricogne et al., 2017) and Phenix.refine (Liebschner et al., 2019). Model building was carried out in Coot (Emsley et al., 2010). TLS parametrization with one set of TLS parameters per domain. Automatically determined NCS restraints were applied during initial refinement steps.

QUANTIFICATION AND STATISTICAL ANALYSIS

Cellular assays
For whole blood pSTAT1 inhibition studies with SRF388, the geometric mean fluorescence intensity (gMFI) of PE-pSTAT1 in CD3+ T cells was determined, and the percent inhibition was calculated in FlowJo_V10 Software (Ashland, Oregon). Raw data were normalized to the stimulated control level (0% inhibition) and unstimulated control level (100% inhibition). Percent inhibition values were plotted in GraphPad Prism software and IC50 was determined using a 4-parameter nonlinear regression equation. Data represent the average response of 2–3 individuals (tested in duplicate) for each species.

For IL-27 mediated pSTAT1 blocking experiments with anti-gp130 blocking antibodies, the data represent the mean of 2 technical replicates per data point with error bars indicating SD. These data are representative of two independent experiments with U937 cell line or PBMCs from healthy donors. Samples were analyzed using the FlowJo Software analysis program (TreeStar). Cytokine stimulated conditions represents 0% inhibition and unstimulated conditions represents 100% inhibition.

Bio-layer interferometry
Mouse IL-27 binding to cognate receptors: Data traces were fitted using a 1:1 interaction model to quantify the kinetics (kₐ, kᵈ) and binding affinity (K_D) of the interactions using the Octet Analysis Studio 12.2.1.24 software. For each experiment three technical replicates were performed. The reported K_D, kₐ and kᵈ values represent average values from three technical replicate experiments. SRF388 binding human IL-27 and Species Cross-Reactivity: all data were subtracted from the no analyte reference, analyzed, and fitted using DataAnalysis software v10.0.1.7 (Pall ForteBio; Fremont, CA) using a 1:1 binding model.