Structural basis of human IL-18 sequestration by the decoy receptor IL-18 binding protein in inflammation and tumor immunity

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Human Interleukin-18 (IL-18) is an omnipresent proinflammatory cytokine of the IL-1 family with central roles in autoimmune and inflammatory diseases and serves as a staple biomarker in the evaluation of inflammation in physiology and disease, including the inflammatory phase of COVID-19. The sequestration of IL-18 by its soluble decoy receptor IL-18-Binding Protein (IL-18BP) is critical to the regulation of IL-18 activity. Since an imbalance in expression and circulating levels of IL-18 is associated with disease, structural insights into how IL-18BP competes binding of IL-18 by its cognate cell-surface receptors are highly desirable; however, the structure of human IL-18BP in complex with IL-18 has been elusive. Here, we elucidate the sequestration mechanism of human IL-18 mediated by IL-18BP based on the crystal structure of the IL-18:IL-18BP complex. These detailed structural snapshots reveal the interaction landscape leading to the ultra-high affinity of IL-18BP toward IL-18 and identify substantial differences with respect to previously characterized complexes of IL-18 with IL-18BP of viral origin. Furthermore, our structure captured a fortuitous higher-order assembly between IL-18 and IL-18BP coordinated by a disulfide-bond distal to the binding surface connecting IL-18 and IL-18BP molecules from different complexes, resulting in a novel tetramer with 2:2 stoichiometry. This tetrapartite assembly was found to restrain IL-18 activity more effectively than the canonical 1:1 complex. Collectively, our findings provide a framework for innovative, structure-driven therapeutic strategies and further functional interrogation of IL-18 in physiology and disease.

Originally discovered in mice as interferon-γ-inducing factor, Interleukin-18 (IL-18) was found to be a potent inducer of interferon-γ (IFN-γ) production in synergy with IL-12 upon exposure to intracellular pathogens (1–3). It was subsequently cloned and identified as an 18-kDa cytokine belonging to the IL-1 cytokine family and renamed to IL-18 (4). Similar to other IL-1 family cytokines, IL-18 is expressed as an inactive precursor protein that requires cleavage by caspase-1 for full biological activity (5, 6). While approximately 80% of pro-IL-18 is retained intracellularly, the rest is released by macrophages/monocytes and dendritic cells after maturation by caspase-1 (7, 8). Mature IL-18 initiates signaling by establishing a tripartite complex with IL-18 receptor alpha (IL-18Ra) and IL-18 receptor beta (IL-18Rβ), the latter also known as IL-18 receptor accessory protein and IL-1R7. The heterodimerization of the receptors’ intracellular Toll/IL-1 receptor domains triggers recruitment of the adapter protein myeloid differentiation primary response 88 (MyD88), which further activates the downstream signaling cascade through IRAK-1/4, TRAF6, and NFKB (9–12). IL-18 is mainly involved in the activation of NK and T helper 1 cells and IFN-γ production in response to intracellular bacteria or viruses (13, 14).

Due to its highly proinflammatory and pleiotropic activity, IL-18 signaling is highly regulated at many levels to prevent uncontrolled inflammation. Akin to other IL-1 family cytokines, this regulation is achieved through gene regulation (15), caspase-1 activation (5, 6), and importantly, is also mediated by a soluble decoy receptor (16). Once IL-18 is activated and released from immune cells, its availability is mainly regulated by sequestration by IL-18-binding protein (IL-18BP), a dedicated soluble decoy receptor-like protein capable of blocking the biological activity of IL-18 (17). Even though IL-18BP functions as a decoy receptor, it is not homologous to any extracellular domain of the IL-18 receptors and is encoded as a separate gene (18), an oddity in the cytokine field (19). However, bioinformatic approaches have proposed that IL-18BP is evolutionary related to TIGIRR-1 (aka IL-1R9) and is thus regarded as a member of the IL-1 receptor family. In addition, sequence and functional homology lead to the identification of IL-18BP orthologs in all orthopox viruses (20, 21), including ectromelia poxvirus IL-18-binding protein (ectvIL-18BP) and yaba-like disease virus IL-18-binding protein (yldvIL-18BP) (22, 23), that act as virulence factors by attenuating immune responses mediated by IL-18.

Not only is human IL-18BP present in the serum at a 20-fold molar excess (24), it also has an exceptionally high affinity (Kd ~ 300 pM) for IL-18 (25), in contrast to IL-18α’s

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affinity toward hIL-18, which is reported to be several orders of magnitude weaker ($K_D \sim 69 \text{nM}$) (26). Intriguingly, the affinity of viral IL-18BPs for human IL-18 is considerably lower than human IL-18BP ($K_D \sim 1 \text{nM}$), although they display picomolar affinity toward mouse IL-18 (25). As a result, IL-18 is sequestered by IL-18BP under homeostatic conditions, thereby preventing it from signaling through its receptors and evoking unwanted proinflammatory responses. Interestingly, IL-18-induced IFN-γ upregulates IL-18BP expression, creating a negative feedback loop to dampen and resolve inflammation (27, 28). Several (auto)immune diseases have been associated with increased levels of IL-18 such as rheumatoid arthritis (RA) (29, 30), Crohn’s disease (CD) (31, 32), and systemic lupus erythematosus (33–35). As IL-18BP has a high IL-18 sequestration capacity (35), the balance between IL-18/IL-18BP and the concentrations of free IL-18, instead of total IL-18, are more relevant to measure to evaluate inflammatory responses (36). Elevated levels of free IL-18 have been identified in hyperinflammatory diseases such as macrophage activation syndrome and systemic juvenile idiopathic arthritis (28, 37). Furthermore, in mouse models of macrophage activation syndrome, IL-18BP-deficient mice developed more severe disease manifestations, pointing to the crucial role of IL-18BP activity in this setting (28). In addition, elevated levels of IL-18 in blood and bronchoalveolar lavage fluid from coronavirus patients have been shown to correlate with COVID-19 disease severity and worse clinical outcomes (38–40).

Unsurprisingly, IL-18-neutralizing antibodies or recombinant IL-18BP have been successfully used to mitigate IL-18 related pathologies (41, 42). Clinical trials using recombinant hIL-18BP (Tadekinig α) for adult-onset Still’s disease (AOSD), RA, and plaque psoriasis show promising safety and indicative signs of efficacy in patients with AOSD (43, 44). On the other hand, administration of recombinant IL-18 in mouse tumor models was shown to elicit favorable antitumoral effects in synergy with chimeric antigen receptor T and immune checkpoint inhibitors (45). Whereas such findings provided a strong rationale for the therapeutic potential of IL-18 in cancer (46), its efficacy has not lived up to expectations (47), the culprit being the concomitant increase in the serum concentration of IL-18BP by up to 100-fold in patients (46, 48).

To aid further advancements in the mechanistic interrogation of IL-18 sequestration by IL-18BP and the therapeutic targeting of hIL-18 and hIL-18BP in infectious diseases, inflammation, and cancer, structural details of their complex and of interaction interface to high resolution would be opportune. Here, we present the crystal structure of human IL-18 in complex with human IL-18BP. This study shows that hIL-18BP binds hIL-18 at the same epitope as IL-18/IL-18α and viral IL-18BPs using a large hydrophobic patch flanked by two tightly fitting hydrophobic pockets complemented by salt bridges. A crucial advance in our crystallization efforts was the elimination of the flexible N-terminus and minimization of heterogenous glycosylation patterns of hIL-18BP while retaining functional and biological activity. Moreover, we discovered a novel disulfide-linked interface resulting in an unexpected tetrameric assembly of hIL-18 and hIL-18BP. The structural model presented here is imperative to validate previous models, map key differences between human and viral orthologs of IL-18BP, and allows advancements in the design of novel inhibitors or antagonists.

Results

**Human IL-18BP can be truncated to a bioactive core structure with reduced glycosylation**

To enable structural studies of the human IL-18BP:IL-18 complex by X-ray crystallography, we initially considered clinical grade human IL-18BP (Tadeking α) that was used in a phase 2 clinical trial to evaluate the safety and efficacy of IL-18BP in AOSD (43). However, due to the extensive glycosylation of this protein and the need to create glycaneengineered protein amenable to structural studies by X-ray crystallography, we produced full-length hIL-18BP in transiently transfected HEK293S MGAT1−/− cells allowing for N-linked glycosylation as Man$_2$GlcNAc$_2$ glycans (49) and mature hIL-18 in *Escherichia coli*. Using size-exclusion chromatography (SEC) and in-line multi-angle laser light scattering (MALLS), we found that full-length hIL-18BP was heavily glycosylated with nearly 50% of its mass accounted for by glycans (Fig. 1A and Table 1), consistent with predictions (Fig. 2A). Furthermore, the distribution of mass across the SEC-MALLS peak for full-length hIL-18BP, the diffuse electrophoretic mobility of the purified protein in SDS-PAGE (Fig. 1, A and B), and large shifts in mass upon treatment with endoglucanases and O-glycosidases suggested heterogeneous glycosylation. Based on our experience with producing secreted glycoproteins with N-linked glycosylation and similar protein families in the HEK293T and HEK293S MGAT1−/− cell lines for structural biology (50–53), we reasoned that this most likely originates from O-glycans and possible differences in N- and O-glycosylation site occupancy. Initial crystallization trials using purified hIL-18BP$_{FL}$:IL-18 complex containing glycanshaved hIL-18BP$_{FL}$ and displaying apparent stoichiometry of 1:1 as evaluated by SEC-MALLS did not lead to candidate crystallization conditions even when employing hIL-18BP$_{FL}$:IL-18 complex up to 30 mg/ml. Such apparent high solubility during crystallization trials was deemed to be consistent with the observed extensive and heterogeneous glycosylation of hIL-18BP$_{FL}$ (Fig. 1A and Table 1). Therefore, we sought to produce alternative versions of hIL-18BP that would be more amenable to structural studies. Sequence alignment of hIL-18BP with homologous viral IL-18BPs, for which structural information is available (22, 23), suggested that the expected core beta-sandwich domain of hIL-18BP (residues 63–171) might be flanked by N- and C-terminal stretches (residues 31–62 and 170–194, respectively) bearing intrinsic disorder and the majority of the predicted O-glycosylation sites (Fig. 2, A and B).

To this end, we produced hIL-18BP lacking the N- and/or C-terminal regions in HEK293S MGAT1−/− cells. We found that only hIL-18BP truncated at the N-terminus (residues 63–194), hereafter termed hIL-18BP$_{ΔN}$, could be sufficiently
Figure 1. Biochemical characterization and functional activity of human IL-18 and IL-18BP. A, SEC-MALLS analysis of hIL-18BPFL, hIL-18BPΔN, and hIL-18BPΔN-EH protein (complex), respectively, yielding less glycosylated and more homogenous material for crystallization purposes. B, SDS-PAGE of hIL-18BPFL, hIL-18ΔN, and hIL-18BPΔN-EH show decline in total protein and glycan mass caused by removal of O-glycosylated N-terminus and shaving of N-glycans by Endoglycosidase H (EH). SDS-PAGE of hIL-18 and hIL-18:hIL-18BPΔN-EH complex show presence and purity of both proteins. C, SEC-MALLS analysis of hIL-18:hIL-18BPΔN-EH complex prior to crystallization allow accurate stoichiometry determination (A) and (C) full lines show the SEC retention profile of samples (~0.5–1 mg/ml) detected by UV (left axis, 280 nm). Interrupted lines show the molecular weights in kDa of the sample over the course of the SEC peak, split up by protein conjugate analysis in total (dashed and dotted), protein (dashed), and glycan (dotted) mass (right axis). D, isothermal titration calorimetry (ITC) of hIL-18 in hIL-18Rα, hIL-18BPFL, and hIL-18BPΔN-EH which shows recombinantly produced hIL-18 and hIL-18BPΔN-EH are both functionally competent.
produced for structural studies. SEC-MALLS analysis of hIL-18BP AN revealed the loss of 3 kDa of protein mass and 5.5 kDa of glycans (Fig. 1A and Table 1), confirming that the N-terminus of hIL-18BP is heavily glycosylated. Additional trimming of N-linked Man 5 GlcNAc2 glycans using Endoglycosidase H (Endo H) resulted in a further loss of 2.5 kDa of N-linked glycans (Fig. 1, A and B).

We initially verified the functional activity of hIL-18BP AN treated with Endo H (IL-18BP AN-EH) in terms of its ability to sequester hIL-18 in stoichiometric and high affinity. Indeed, SEC-MALLS analysis showed that hIL-18BP AN-EH can establish a monodisperse complex with hIL-18 according to a 1:1 stoichiometry (Fig. 1C and Table 1). Using isothermal titration calorimetry, we compared the thermodynamic binding profiles and affinities of hIL-18BP FL, hIL-18BP AN-EH, and the hIL-18 cognate receptor (IL-18Ra) to hIL-18 (Fig. 1D). Our data show that hIL-18BP AN-EH and hIL-18BP FL are virtually indistinguishable from each other in their thermodynamic binding profiles and sub-nM affinities toward hIL-18. In contrast and consistent with previously published data (26), human hIL-18Ra binds to hIL-18 with lower affinity (K D = 4.7 nM) than hIL-18BP. However, the binary complex affinity measured here is markedly higher than previously reported affinities (K D ~60 nM) (26) by surface plasmon resonance.

Finally, we interrogated the biological activity of hIL-18BP AN-EH by using the hIL-18-responsive macrophage cell line KG-1, the activity of which is manifested by NFkB signaling and proinflammatory cytokine and chemokine production (54). In this context, we measured the amount of IL-8 secreted from KG-1 cells upon hIL-18 stimulation and found that both hIL-18BP FL and hIL-18BP AN-EH display comparable inhibitory activities. (Fig. 1E).

Structural mimicry underlies the sequestration of human IL-18 by IL-18BP

Structural insights into the sequestration of human IL-18 by its cognate decoy receptor IL-18BP had remained elusive despite the growing importance of IL-18BP in physiology and disease. Using the truncated and glycan-shaved hIL-18BP FL, we purified crystallization-grade hIL-18:hIL-18BP AN-EH complex and determined the crystal structure of the complex to 1.8 Å resolution by molecular replacement using the crystal structure of ectlIL-18BP (23) (pdb 3f62) as a search model (Fig. 3A and Table 2). Crystallographic refinement and the quality of the ensuing electron density maps were enhanced after correcting the X-ray data for data anisotropy using the STARANISO server (55). The crystal asymmetric unit contains one copy of the 1:1 complex and reveals how hIL-18BP uses the side of its h-type immunoglobulin β-sandwich fold to bind the β-trefoil structure hIL-18 (Fig. 3A). hIL-18BP harbors two internal disulfide bridges connecting strands A and B (Cys86-Cys150), and B and F (Cys64-Cys89). In addition, three predicted N-glycan sites are occupied by GlcNAc at residues Asn79, Asn103, and Asn147. Our model for hIL-18BP starts at the beginning of the hIL-18BP AN, construct that is, Gln63, however no density was observed past Ala170 in the crystal solvent channels, consistent with the predicted disorder of the C-terminal tail (Figs. 3C and 2B). Human IL-18 features its signature β-trefoil fold and closely resembles the structure of unbound hIL-18 (pdb 3wo2) (all-atom r.m.s.d=1.8 Å). Structure-based sequence alignments of human IL-18BP and structural superpositions against the two structurally characterized viral IL-18BP, ectlIL-18BP and yldvIL-18BP, and domain 3 of hIL-18Ra (hIL-18RaD3) establish the strong conservation of the adopted fold, albeit with major variations in the CD loop mediating hIL-18 binding and AB loop (Fig. 3, B–D). Importantly, these analyses reveal that structural mimicry and direct steric competition underlie the sequestration of human hIL-18 by hIL-18BP (Fig. 3C).

Specifically, human IL-18BP covers the top of the hIL-18 β-barrel with one side of its β-sandwich scaffold composed of strands C, G, and F and is slightly inclined to enable protrusion of loops FG and BC into the barrel. Additionally, residues from the CD loop and D strand mediate important interactions on the other side of the sheet (Fig. 3A). Considering the plethora of structural information on human/viral IL-18BPs and human IL-18Ra in complex with hIL-18,

**Table 1**

Molecular weights of recombinant proteins used in this study and IL-18 receptor complexes as estimated via SEC-MALLS and binding parameters for the binding of hIL-18 to receptors as determined by ITC

| Protein (complex)       | Molecular weight determined by MALLS | Binding to hIL-18 determined by ITC |
|-------------------------|--------------------------------------|-----------------------------------|
|                         | Total MW ± SD (kDa)                  | Protein MW ± SD (kDa)             | glycan MW ± SD (kDa) | K D (95% CI) (nM) | ΔH (95% CI) (kcal/mole) |
| hIL-18                  | 18.2 ± 0.5                           | 17.6 ± 0.1                        | 14.8 ± 0.6           | 19.32 (−∞ to 0.245) | −19.32 (−∞ to 20.32) |
| hIL-18BP FL             | 17.6 ± 0.5                           | 14.65 ± 0.06                      | 9.3 ± 0.1            | n.d. (−∞ to 0.817) | −18.13 (−∞ to 19.36) |
| hIL-18BP AN             | 14.8 ± 0.2                           | 14.42 ± 0.02                      | 6.71 ± 0.08          | n.d. (−∞ to 4.79)  | −26.63 (−∞ to 27.77) |
| hIL-18BP AN-EH          | 14.4 ± 0.02                          | 38.80 ± 0.06                      | 6.9 ± 0.2            | 5.24 (7.94 to 3.38) | −26.63 (−∞ to 27.77) |
| hIL-18Ra                | 35.4 ± 0.2                           | −                        | −                        | −                        | −                        |

Abbreviations: MW, Molecular weight; CI, Confidence interval; MALLS, Multi-Angle Laser Light Scattering; ITC, Isothermal Titration Calorimetry.

Bottom plots show the raw baseline-subtracted thermograms while top plots show integrated heats (blue dots) with fitted isotherm (red line). Diagrams in the isotherm plot show which protein was in the cell or syringe and what the expected final complex looks like. E, bar plot of IL-8 levels in conditioned media after hIL-18 stimulation in presence or absence of hIL-18BP FL or hIL-18BP AN-EH. Recombinant IL-18 was preincubated for 1 h at 37 °C with the indicated concentrations of full-length hIL-18BP or hIL-18BP AN-EH. KG-1 cells were left untreated (UT) or stimulated with IL-18 (purple) (0.5 nM final concentration) ± IL-18BP (teal and orange), as indicated. After 18 h, IL-8 concentration in cell culture supernatants was measured by ELISA. Error bars display standard deviation (SEM). IL-18, interleukin-18; SEC, size-exclusion chromatography; MALLS, multi-angle laser light scattering.
curation and comparison of the observed interaction interfaces
to distil key structure-function insights presents with consid-
erable challenges. At the same time, annotation of amino acid
positions that have been interrogated by mutagenesis would
also be desirable. Together, such analyses could lead to a
rationale for the exquisitely ef
fi
fi cient sequestration of hIL-18 by
hIL-18BP and might further fuel protein engineering ap-
proaches to modulate this sequestration potential. Therefore,
we sought to develop a representation scheme that would
allow effective parallel comparison of all structural data
available together with mutagenesis data (Fig. 4
B
). The binding
interface can be subdivided into three sites: site A consists of
complementary hydrophobic patches on both hIL-18 and hIL-
18BP. Sites B and C each engage a phenylalanine on hIL-18BP,
Phe106, and Phe95, respectively, to protrude into a hydro-
phobic cavity on hIL-18 sealed by salt bridges and hydrogen-
binding interactions (Fig. 4
A
). Sequence alignments of
hIL-18BP orthologs among vertebrates (Fig. 5
A
) allow map-
ning of highly conserved residues on the surface of hIL-18BP
that mediate sequestration of hIL-18 and shows that this re-
gion of hIL-18BP is the most conserved compared to any other
part of the decoy receptor (Fig. 5
B
). This suggests that the
observed human IL-18BP:IL-18 complex can serve as a
structural representative for all such complexes across species.
When comparing the interface of human IL-18BP with its viral
counterparts, these phenylalanine residues are largely
conserved, except for yldvIL-18BP, which contains a threonine
instead of phenylalanine at site B. The most striking difference
is the lack of salt bridges in viral IL-18BPs at site C.
Conversely, human IL-18Rα does form these salt bridges but
lacks the phenylalanine residues protruding into the hydro-
phobic cavity of hIL-18, while also lacking another hydro-
phobic substitute. At site B, hIL-18Rα also lacks the
phenylalanine residue but substitutes it for a methionine to
fi
fi ll
the hydrophobic pocket (Fig. 4
B
). The absence of these
phenylalanine residues in sites B and C may explain the higher
affinity of human IL-18BP to IL-18 than its receptor. Overall,
site A is very similar for all interfaces with mainly conservative
substitutions of key interacting residues. However, like
IL-18Rα, but in contrast to the viral IL-18BPs, human IL-18BP
exploits additional interactions via two side-chain-to-main-
chain hydrogen bonds at site A.

A disulfide-linked interaction site mediates a novel
higher-order IL-18:IL-18BP complex

An intriguing feature of the IL-18:IL-18BP complex con-
cerns an unexpected disulfide bridge between Cys74 of hIL-18
in the canonical IL-18:IL-18BP complex and Cys131 of
IL-18BP participating in a symmetry-related complex. This
results in a covalent tetrameric assembly with a 2:2 stoichi-
ometry and two-fold symmetry (Fig. 6
A
). We note that such
higher-order assembly was not observed in the preparatory
stages of precrystallization samples as assessed by SEC-
MALLS or SDS-PAGE (Fig. 1,
B
and
C
). However, the sam-
ple was concentrated to 30 mg/ml prior to crystallization. To
verify whether such a higher-order complex originated during this final protein concentration step, the concentrated sample was diluted back to 0.5 mg/ml and analyzed by SDS-PAGE and SEC-MALLS. SDS-PAGE shows a band at ∼38 kDa which can be resolved to the constituent protein components upon the addition of the reducing agent DTT prior to loading the sample, indicating the presence of a disulfide linked dimer (Fig. 6B). We confirmed by mass spectrometry that both hIL-18 and hIL-18BP were present in this disulfide linked protein adduct, verifying a heterodimer as opposed to a homodimer of either. Additionally, SEC-MALLS shows that more than 90% of the previously concentrated sample now exists as a tetramer of 74 kDa, rather than the expected canonical IL-18:IL-18BP complex of 37 kDa (Fig. 6C). Protein conjugate analysis of SEC-MALLS data validated the presence of both hIL-18BP and hIL-18 in the complex with a 2:2
with IL-18BP molecules. and is maintained by a several hydrophobic residues on both side of the beta-sandwich compared to the canonical interface part of a 310 helix and is not surface exposed (Fig. 6 involved in the observed novel disul in complex with its decoy and signaling receptors, the cysteine molecular masses. glycans would not be compatible with their respective mo-

Table 2

| Data collection | Human IL-18:hIL-18BP_{AN} EH | Human IL-18:hIL-18BP_{AN} EH corrected for diffraction-anisotropy |
|-----------------|-------------------------------|---------------------------------------------------------------|
| Wavelength (Å)  | 0.9762                        | 0.9762                                                        |
| Resolution range (Å) | 59.39–1.80 (1.87–1.80)     | 59.39–1.80 (1.85–1.80)                                        |
| Space group     | C 1 2 1                       | C 1 2 1                                                      |
| Unit cell (a, b, c (Å), α, β, γ (°)) | 109.82 44.52 60.28 90 99.86 90 | 109.82 44.52 60.28 90 99.86 90                                |
| Total reflections | 183,218 (17,814)              | 140,440 (6871)                                               |
| Unique reflections | 26,507 (2546)                 | 20,705 (1035)                                               |
| Multiplicity    | 6.9 (7.0)                     | 6.6 (6.6)                                                   |
| Completeness (%) | 98.27 (96.15)                 | 77.2 (49.6)                                                 |
| Mean I/σ(I)     | 15.67 (1.49)                  | 18 (2.9)                                                   |
| Wilson B-factor (Å²) | 36.35                      | n.a.                                                        |
| R-meas          | 0.053 (1.327)                 | 0.048 (0.680)                                              |
| R-pim           | 0.020 (0.495)                 | 0.018 (0.258)                                              |
| CC1/2           | 0.999 (0.896)                 | 0.999 (0.898)                                              |
| CC*             | 1.000 (0.972)                 | n.a.                                                        |
| Refinement (59.39–1.8 Å) | 26,368 (2525)             | 20,691 (1306)                                              |
| Reflections used in refinement | 2640 (248)                 | 2062 (130)                                                  |
| Reflections used for R-free | 0.207 (0.531)/0.230 (0.586) | 0.188 (0.264)/0.217 (0.320)                                  |
| CC (work)/CC (free) | 0.968 (0.860)/0.959 (0.812) | 0.874 (0.689)/0.858 (0.640)                                  |
| Number of nonhydrogen atoms | 2309                        | 2309                                                        |
| Macromolecules  | 2136                          | 2136                                                        |
| Ligands         | 42                            | 42                                                          |
| Solvent         | 131                           | 131                                                         |
| RMS bonds (Å)/angles (°) | 0.011/1.34                  | 0.011/1.34                                                  |
| Ramachandran favored (%) | 98.05                      | 98.05                                                       |
| Ramachandran allowed (%) | 1.95                       | 1.95                                                        |
| Ramachandran outliers (%) | 0                           | 0                                                           |
| Rotamer outliers (%) | 0                           | 0                                                           |
| Clash score     | 3.48                          | 3.48                                                        |
| Average B-factor (Å²) | 34.68                      | 34.68                                                       |
| Macromolecules  | 34.03                         | 34.03                                                       |
| Ligands         | 66.67                         | 66.67                                                       |
| Solvent         | 35.15                         | 35.15                                                       |

Values in parentheses correspond to the highest resolution shell (1.87–1.80 Å).

| R-work/R-free | 0.207 (0.531)/0.230 (0.586) | 0.188 (0.264)/0.217 (0.320) |
|---------------|-----------------------------|----------------------------|
| CC (work)/CC (free) | 0.968 (0.860)/0.959 (0.812) | 0.874 (0.689)/0.858 (0.640) |

Interestingly, in the structure of unbound hIL-18 and when in complex with its decoy and signaling receptors, the cysteine involved in the observed novel disulfide bond with IL-18BP is part of a 310 helix and is not surface exposed (Fig. 6A, left panel). This region in inbound IL-18 is rather amphipathic in terms of physicochemical properties and interacts with several detergent molecules (CHAPS) in pdb 3wo2 (26). Thus, to participate in the observed disulfide bond, the cysteine has flipped outward and is accompanied by a structural rearrangement thereby disrupting the helical structure (Fig. 6A, left panel). Interestingly, an ensemble of structural models of IL-18 determined by NMR has shown this helix to be very flexible (56), which would favor such dynamics. We note that for the reported crystals structure of hIL-18 complexed with ectvIL-18BP and yldvIL-18BP (pdb 3f62 and 4eee respectively), all free cysteines were mutated to serine, thereby excluding the possibility of such a disulfide linkage, although this helix/loop segment was not modeled due to its apparent flexibility. Surrounded the cysteine disulfide bridge is a novel interface mediated by the beta strands E, B, D, and D’ of the opposite side of the beta-sandwich compared to the canonical interface and is maintained by a several hydrophobic residues on both molecules.

Because this hetero-tetramer was only initially observed with IL-18BP_{AN-EH} lacking the N-terminal and N-glycans, we also investigated if a similar higher-order complex could be formed with IL-18BP_{FL} or endogenously glycosylated IL-18BP_{ΔN}. Full-length IL-18BP was complexed with IL-18, concentrated to 0.5 mg/ml and analyzed by SEC-MALLS. No tetrameric complex was observed using IL-18BP_{FL}, either in its glycosylated or deglycosylated form, suggesting that removal of the N-terminal segment of IL-18BP promotes the formation of this hetero-tetrameric complex (Fig. 6C). We note that Cys51 is part of the N-terminal truncation and likely pairs up with Cys131 in the full-length protein, thus preventing heterotetrameric complex formation. Endogenously glycosylated IL-18BP_{ΔN}, without Endo H treatment, also formed a tetrameric complex with IL-18 at high concentrations, albeit at a lower rate than IL-18BP_{AN-EH} (Fig. 6D).

The presence of a cysteine disulfide bridge linking IL-18BP_{AN} and IL-18 distal to the binding interface in the hetero-tetrameric assembly observed here may result in better sequestration and inhibition of IL-18 than in the canonical 1:1 complex. Furthermore, the site where disulfide-linked IL-18BP engages IL-18 would also sterically clash with the eventual binding site of IL-18 to domain 1 of IL-18Rα. To test this, both the heterodimeric or heterotetrameric complex of IL-18BP_{AN-EH} and IL-18 was purified and used to stimulate KG-1 cells. While both the dimeric and tetrameric complex inhibited IL-18 activity compared to IL-18 alone, the tetra-
teramic assembly restrained IL-18 activity more effectively than the dimeric complex, resulting in less inflammatory cytokine production from KG-1 cells (Fig. 6E).
Figure 4. Interaction interface between human IL-18 and human IL-18BP and comparisons with human IL-18 receptor and viral receptor decoys.

A, top right panel shows an overview of interacting structures of hIL-18BPΔN-EH (cartoon, orange) with hIL-18 (surface, purple). Structures of hIL-18BPΔN-EH shown consist out of the bottom half of the beta-sandwich, with the top half and noninteracting loops hidden. Top left, bottom left, and bottom right panels show zoomed-in views of the three main interaction sites B, A, and C, respectively, with hIL-18BPΔN-EH and hIL-18 shown in cartoon representation with a transparent surface overlay for hIL-18. Key residues are shown in stick representation colored by element (blue: nitrogen, red: oxygen, yellow: sulfur, white: polar hydrogens, orange/purple: carbon). Nonpolar hydrogens are hidden. B, diagrams show simplified interactions at site A, B, and C of hIL-18 (purple) with hIL-18BP (orange), viral ectxIL-18BP and ylvIL-18BP (cyan), and domain 3 of hIL-18Ra (dark blue). The interaction between hIL-18 and hIL-18BP consists of three sites: A, B, and C. Site A consists of interlocking rows of hydrophobic residues. Site B consists of a phenylalanine flanked by hydrophobic side chains supplemented with two salt bridges. Site C consists of a phenylalanine residing in a large pocket lined by hydrophobic residues supplemented by two salt bridges. Notable differences with ectxIL-18BP and ylvIL-18BP are missing salt bridges at site C, even though a capable side chain is present for ectxIL-18BP. ylvIL-18BP also has a threonine replacing the phenylalanine at site B. hIL-18Ra-D3 does have the salt bridges at site C but is lacking the phenylalanine with no substitution, while at site B, the phenylalanine is replaced by a methionine. Residues marked with a red asterisk have been reported to have reduced affinity after certain mutations. Residues marked with a green asterisk have been reported to not be affected by certain mutations. Mutagenesis data were
Discussion

Over the past two decades and in the absence of an experimentally determined structure for the human IL-18:IL-18BP complex, numerous studies have used orthogonal methods to predict how human IL-18BP interacts with human IL-18 to efficiently sequester and prevent signaling. To date, structural features and binding interfaces between human IL-18 and human IL-18BP have been inferred from computational models (44), human and viral mutagenesis studies (45–49), and homologous structures of viral IL-18BPs in complex with human IL-18 (22, 23). Additionally, the ternary assembly of human IL-18 in complex with its cognate receptors, IL-18Rα and IL-18Rβ (26), has shed some light on the potential for competitive binding of human IL-18BP. The high-resolution structural analysis of human IL-18BP bound to IL-18 presented here is imperative to further understand and exploit the ultra-high affinity binding mode that characterizes this unique decoy receptor-like protein (22, 23). Human IL-18BP binds the same epitope of hIL-18 used by viral IL-18BPs to compete with the third ectodomain of hIL-18Rα. The interface (site A) predominantly consists of two large, complementary hydrophobic patches, mimicking the landscape of hydrophobic peaks and troughs on each side of the interface, supplemented with two side-chain-to-main-chain hydrogen bonds. Comparing the structure of hIL-18BP with its viral orthologs confirms that this large hydrophobic patch on site A is highly conserved.

yldvIL-18BP contains some additional hydrophobic residues (Ile78 and Leu80) on the DE loop interacting with hIL-18 (Leu5 and Glu6) at the perimeter of the interface. In all other structures, except yldvIL-18BP, this loop is constrained as an additional βD0 strand. On each side of the hydrophobic patch, a phenylalanine on hIL-18BP is tightly inserted inside hydrophobic cavities on hIL-18 and additionally locked down by salt bridges (site B and C, Fig. 4B). Mutating either phenylalanine residues to alanine reduces the ultra-high picomolar affinity of hIL-18BP down to low-nanomolar affinity (22, 23, 57). Mutations of the corresponding residues in viral IL-18BPs have a similar effect, highlighting the importance of these specific residues for binding. Interestingly, in hIL-18Rα, the corresponding residue is either substituted by methionine (site B) or completely lacking (site C), although site B is flanked by additional hydrophobic residues residing on hIL18Rα’s longer CD0 loop. The absence of these seemingly critical phenylalanine residues may, in part, explain the higher affinity of hIL-18BP than its receptor. Similarly, mutating Glu108 and Glu106 on hIL-18BP involved in the salt bridges at site B accordingly resulted in a lower affinity, as well as mutating Lys53 on hIL-18 which is located on the other end of the salt bridges (58, 59). Mutations in site C particularly have not been reported, because in both viral structures the corresponding residues (Glu48 in ectvIL-18BP and His51 in yldvIL-18BP) are not involved in salt interactions with hIL-18.
Figure 6. Tetrapartite assembly of human IL-18 and human IL-18BP. A, two complexes of hIL-18:hIL-18BPΔN-EH are linked through an intermolecular disulfide bridge between hIL-18 and hIL-18BPΔN-EH from neighboring symmetric related unit forming a hetero-tetrameric assembly with a novel interface. Middle panel shows an overview of the assembly using cartoon representation of hIL-18 (purple or teal) and hIL-18BPΔN-EH (orange) with the disulfide links involved shown in yellow stick representation. Left panel shows a zoomed-in view with an aligned overlay of the 310 α-helix from a crystal structure of unbound hIL-18 (teal, pdb 3wo2 (26)), showing disruption of the helix upon formation of the disulfide link. The right panel displays the novel interface surrounding the disulfide link with hIL-18 shown in surface representation (purple, pdb 7al7) and hIL-18BPΔN-EH in cartoon representation (orange, pdb 7al7) with key residues displayed in stick representation. Secondary structure elements and key residues are labeled accordingly. B, SDS-PAGE analysis of hIL-18:hIL-18BPΔN-EH complex sample after concentration to 30 mg/ml prior to crystallization and diluting back to 0.5 mg/ml with oxidizing (H2O2), reducing (DTT), or no agents added. Reduction results in dissociation of the otherwise disulfide-linked tetramer. C, SEC-MALLS analysis of hIL-18:hIL-18BPΔN-EH, and hIL-18:hIL-18BPΔN-EH complex before and after concentration to 30 mg/ml prior to and diluting back to 0.5 mg/ml. Line plots show the SEC retention profile of samples detected by UV (left axis). Interrupted lines plot the molecular weights of the sample over the course of the SEC peak, dissected by protein conjugate analysis in total (dashed and dotted), protein (dashed), and glycan (dotted) mass (right axis). D, SEC-MALLS analysis of hIL-18:hIL-18BPΔN and hIL-18:hIL-18BPFL complex sample after concentration to 30 mg/ml and diluting back to 0.5 mg/ml. Line plots show the SEC retention profile of samples detected by UV (left axis). Interrupted lines plot the molecular weights of the sample over the course of the SEC peak, dissected by protein conjugate analysis in total (dashed and dotted), protein (dashed), and glycan (dotted) mass (right axis). E, bar plot of IL-8 levels (y-axis, pg/ml) in conditioned media after human IL-18 stimulation in presence or absence of dimeric or tetrameric IL-18BPΔN-EH:IL-18 complex. Recombinant IL-18 was preincubated for 1 h at 37 °C with the indicated concentrations of full-length IL-18BP or IL-18BPΔN-EH. KG-1 cells were left untreated (UT) or stimulated with IL-18 (purple) (0.5 nM final concentration) ± IL-18BP (teal and orange), as indicated. After 18 h, IL-8 concentration in cell culture supernatants was measured by ELISA. Error bars display standard deviation (SEM). IL-18, interleukin-18; SEC, size-exclusion chromatography; MALLS, multi-angle laser light scattering.
bridge formation. Overall, hIL-18BP utilizes a unique constellation of key residues contributing to ultra-high affinity binding to IL-18, however further investigation is required to determine binding hotspots important for this interaction. Whether the lower affinity of viral IL-18BPs is advantageous or disadvantageous for their function remains unclear.

Unexpectedly, we observed a higher order assembly of IL-18 and IL-18BP in our crystallographic model, coordinated by an otherwise buried cysteine in IL-18, disrupting an alpha helix in the process. Using SEC-MALLS and mass spectrometry, we confirmed the formation of a heterotetramer, consisting of two complexes of IL-18:IL-18BP, with an additional interaction interface distal to the canonical-binding site of IL-18BP. Interestingly, this heterotetrameric complex restrained IL-18 activity more effectively than the canonical dimeric 1:1 complex. Given that high concentration and the removal of the N-terminal region appear to be required, it is likely that the observed oligomeric state is not biologically relevant. However, this fortuitous observation suggests that this interface may be amenable to further engineering to increase the sequestration potency of IL-18BP, possibly in combination with domains 1 and 2 of IL-18Rα. It brings to light plasticity in IL-18, the possible reactivity of Cys131 in IL-18BP, and the possible utilization of a hydrophobic patch at opposite ends to the IL-18:IL-18BP interface to create a new interaction interface. An analogous yet distinct bivalency has been observed in viral yldvIL-18BP. This ortholog lacks a highly conserved phenylalanine residue in site B, however it compensates for this by forming a 2:2 complex with IL-18, driven by disulfide-linked IL-18BP homodimerization (23). However, we note that the 2:2 heterotetrameric complex seen here for human IL-18BP engages a separate interface and is driven by an additional interaction with IL-18.

The structural model presented here will further facilitate the structure-driven development of novel therapeutics to either disrupt or mimic hIL-18BP binding. Aberrant IL-18 signaling has been implicated in numerous inflammatory diseases including RA, AOSD, systemic lupus erythematosus, sickle cell disease, and more recently, the inflammatory phase of coronavirus infection (28, 29, 36, 37, 60). Coronavirus disease (COVID-19), caused by the SARS-CoV2 virus, displays a broad range of clinical symptoms, resulting in disease severity extending from mild to fatal outcomes. Accumulating evidence has shown that tissue damage in the later stages of severe COVID-19 infection is driven by cytokine release syndrome, also known as cytokine storm (38, 39, 61). Recent studies have detected elevated levels of IL-18 in patients with moderate and severe COVID-19 infection, which strongly correlated with disease severity (38, 39, 62). Longitudinal analysis of patients infected with SARS-CoV-2 showed IL-18 levels remained elevated in severe COVID-19 patients admitted to ICU, while cytokine levels declined over time in moderately affected patients. Moreover, both IL-18 and IL-18BP have been identified as promising biomarkers to predict disease severity and likelihood of death after SARS-CoV-2 infection (38, 63). Elevated levels of IL-18 were also detected in multisystem inflammatory syndrome and Kawasaki syndrome in children following SARS-CoV-2 infection and may be used as a biomarker to distinguish these diseases from similar hyperinflammatory syndromes (37). Increased IL-18 production has also been associated with activation of cytotoxic mucosal-associated invariant T cells during coronavirus infection, leading to lung tissue damage and increased disease severity (40). These studies suggest that modulation of IL-18 activity may represent a novel therapeutic opportunity for COVID-19, however, no clinical trials with recombinant IL-18BP or IL-18-specific antagonistic have yet been initiated (64, 65). However, because of the pleiotropic activity of IL-18, there will likely be a narrow therapeutic window to exploit the beneficial activity of IL-18 for effective viral clearance, while avoiding aberrant inflammation and tissue damage associated with high IL-18 activity during late-stage infection. In addition, IL-18 has been shown to display potent antitumor activity, however clinical trials using recombinant IL-18 have shown limited efficacy (42). IL-18BP, produced in the tumor microenvironment, has been identified as an ‘immune checkpoint’, hampering the therapeutic application of recombinant IL-18 in cancer (66). Directed evolution of yeast-displayed mouse IL-18 variants identified a decoy-resistant IL-18 that retains signaling activity but escapes inhibition by IL-18BP. This IL-18 variant displayed potent cytotoxic T cell activation, NK cell maturation, and tumor growth inhibition compared to its WT counterpart (66). The high-resolution structural models presented here, in particular the experimentally determined interaction interface of IL-18 and human IL-18BP, will be of crucial importance for additional protein engineering and future drug design for the development of novel therapeutics for use in viral infections, autoimmune and inflammatory diseases, and cancer.

### Experimental procedures

**Plasmids, protein expression constructs, and cell lines**

All constructs were created by a traditional restriction ligation approach. Restriction enzymes, T4 ligase, and Q5 polymerase were purchased from NEB (New England Biolabs). All primers were purchased from IDT (Integrated DNA Technologies). Recombinant DNA was purchased from GenScript. The mature sequence of human IL-18 (residues 37–193, UniProt ID: Q14116) was codon optimized for expression in *E. coli* and purchased in the pUC57 vector from GenScript. The sequence was cloned into the pET42a plasmid (Cat No 70561, Novagen, Merck) in frame with an N-terminal caspase 3-cleavable glutathione S-transferase (GST)-tag (26). The introduction of an extra N-terminal hexahistidine (His6)-tag was performed by PCR. The sequence of human IL-18BP (residues 1–194, UniProt ID: O95998) was purchased in the pUC57 vector from GenScript. The sequence was cloned into frame with a C-terminal caspase 3 site followed by an AviTag and a His tag. The construct was further cloned in frame with an N-terminal chicken RTPμ-like signal peptide sequence (67) that replaced the native signal peptide sequence (residues 1–30). For crystallization purposes, the N-terminus (residues 31–60) was removed by PCR (IL-18BPΔN) and the construct
was cloned in the same pHLSec plasmid containing an N-terminal chicken RTP-like signal peptide sequence and a caspase 3 cleavable Avi-His<sub>6</sub>-tag. All constructs were validated by Sanger sequencing by GATC BioTech before further experiments were performed.

**Expression and purification of recombinant proteins**

BL21(DE3) *E. coli* transformed with plasmid pET42 expressing N-terminally GST- and HIS-tagged human IL-18 were grown at 37 °C in LB medium containing kanamycin (25 μg/ml) as selection marker for the pET42a plasmid (Cat No 70561, Novagen, Merck). When the optical density at 600 nm (A600) reached 0.6, the expression of human IL-18 was induced by addition of IPTG at a final concentration of 1 mM, after which the culture was incubated at 28 °C for 5 h. The bacteria were harvested by centrifugation (7000g for 10 min at 4 °C) and the cellular pellet was stored at −80 °C.

The bacterial pellet was thawed and resuspended in Heps-buffered saline (HBS, 20 mM Hepes, 150 mM NaCl, pH 7.4) with DNase (REF 10104159001, Roche Diagnostics). The cells were lysed by sonication with a Qsonica macrotip sonicator (on-time 4 min; pulse on 30 s; pulse off 30 s; amplitude 70%) while cooled on ice. The lysate was clarified by centrifugation (20,000g for 45 min at 4 °C) and filtration using a 0.22-μm Millipore Steritop Sterile Vacuum Bottle-Top Filter (Thermo Fisher Scientific) and loaded onto a HisTrap HP 5 ml column (GE Healthcare) equilibrated with HBS. The column was washed (HBS, 50 mM imidazole) before eluting the protein (HBS, 250 mM imidazole) following desalting using a HiPrep 26/10 Desalting column (GE Healthcare) to remove imidazole. The His<sub>6</sub>-GST-tag was removed by caspase 3 (produced in-house) cleavage at 37 °C for 1 h. To remove the uncleaved protein and the His<sub>6</sub>-GST-tag, the sample was again loaded onto a HisTrap HP 5 ml column (GE Healthcare). The flow-through was collected and concentrated before injection onto a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences) using HBS as running buffer. Fractions containing human IL-18BP or IL-18BP<sub>AN</sub> were pooled, flash frozen, and stored at −80 °C. The purity of the protein was evaluated on SDS-PAGE stained with Coomassie blue.

**Expression and purification of recombinant human IL-18BP**

Adherent HEK293S MGAT1<sup>Δ</sup> cells (68) were grown in 5-layer cell culture flasks (Falcon Multi-Flask, Corning) in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Thermo Fisher Scientific), supplemented with 10% fetal calf serum (Bodinco). Upon transfection, the growth medium was exchanged for DMEM supplemented with 3.6 mM valproic acid (item 13033, Cayman Chemical Company). Transient expression of human IL-18BP or IL-18BP<sub>AN</sub> was achieved using branched PEI (M<sub>n</sub> ~ 10000, Cat.:40872-7, Sigma-Aldrich) as transfection reagent (67). After 4 days of expression, filtered conditioned medium was loaded onto a 5 ml Complete His-tag Purification Column (Roche Diagnostics). The protein was eluted with 250 mM imidazole in HBS after which the imidazole was removed using a HiPrep 26/10 Desalting column (GE Healthcare). The Avi-His<sub>6</sub>-tag was cleaved by caspase-3 (produced in-house) and the glycos was trimmed by Endo H (produced in-house) at 37 °C for 1 h. The flow-through of the HisTrap HP 5 ml column (GE Healthcare) containing the cleaved protein was concentrated and injected onto a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences) using HBS as running buffer. Fractions containing human IL-18BP or IL-18BP<sub>AN</sub> were pooled, flash frozen, and stored at −80 °C. The purity of the protein was evaluated on SDS-PAGE stained with Coomassie blue.

**Multi-angle laser light scattering**

Purified protein samples at a concentration of 0.5 mg ml<sup>−1</sup> were injected onto a Superdex Increase 10/300 Gl column (GE Healthcare) that was pre-equilibrated with HBS. The column was coupled in line with a UV-detector (Shimadzu), a miniDAWN TROES MALLS detector (Wyatt) and an Optilab T-rEX refractometer (Wyatt). Refractive index increment values (dn/dc) of 0.185 ml g<sup>−1</sup> and 0.155 ml g<sup>−1</sup> were used for protein and glycan analysis, respectively. Bovine serum albumin (Pierce) was used as standard to correct for band broadening. The resulting data was analyzed using the ASTRA6.1 software (Wyatt, v6.1) and errors were calculated in Microsoft Excel.

**Isothermal titration calorimetry**

All proteins were buffer matched in HBS from the same batch using SEC and all the solutions were degassed. Protein concentrations were determined with the NanoDrop 2000 (Thermo Fisher Scientific) using their corresponding extinction coefficients (absorbance of 1%). Experiments were carried out on the MicroCal VP-ITC calorimeter (Malvern Panalytical) at 37 °C. Titrations were always preceded by an initial injection of 3 μl and were carried out using 10 μl injections applied 300 s apart. The sample was stirred at a speed of 300 r.p.m. throughout. The data was recorded using the Origin scientific plotting software (Version 7.0, MicroCal, Malvern Panalytical) and further analyzed using NITPIC (69) and SEDPHAT (70) fitting the data with a “one to one” binding model.

**Bioactivity assays**

KG-1 cells were cultured in RPMI media (Gibco) supplemented with 10% fetal calf serum (Bodinco). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. KG-1 cells were plated at 2 × 10<sup>5</sup> cells per well in 24-well plates. Recombinant IL-18 was preincubated with IL-18BP<sub>FL</sub>, IL-18BP<sub>AN,EH</sub> or complexes thereof as indicated, for 1 h at 37 °C. KG-1 cells were left untreated or stimulated with IL-18 (0.5 nM final concentration) ± IL-18BP, as indicated. Cells were incubated with stimuli for 18 h, and cell culture supernatants were subsequently collected. Culture supernatants were centrifuged for 5 min at 1000g to remove cells and frozen at −80 °C.
Structure of human IL-18 in complex with human IL-18BP

Detection of cytokines by ELISA

Human IL-8 was measured from cell culture supernatants using commercially available specific paired ELISA kits (R&D Systems). Briefly, a 96-well plate was coated with 50 ml of capture antibody per well according to manufacturer’s instructions and incubated overnight at room temperature. Wells were washed three times with 150 ml wash buffer (0.05% Tween-20 in PBS, pH 7.2), then blocked for 1 h in reagent diluent (1% bovine serum albumin in PBS, pH 7.2). Samples were diluted as required in reagent diluent, and 50 ml of each sample was added to wells and incubated for 2 h at room temperature. Appropriate standards were prepared for each ELISA assay according to manufacturer’s instructions. Wells were washed again three times, followed by 50 ml of biotin-conjugated detection antibody incubated for 2 h. Wells were washed three times, and 50 ml streptavidin-HRP was added to wells and incubated for 30 min in the dark. Finally, wells were washed three times, followed by addition of 50 ml TMB substrate solution. The reaction was stopped with 30 ml of 2M sulfuric acid and absorbance was read at 450 nm on an ELISA plate reader (Fluostar). All cytokine assays were carried out using triplicate samples from each culture.

Bioinformatic analyses

All sequence alignments were performed by Clustal Omega (71). Structural alignments were performed in Swiss- pdbViewer (72). Glycan prediction was performed using NetNGlyc (73) and NetOGlyc servers (74). Protein disorder was predicted using IUPred (75). Sequence alignments were visualized by ESPript (76). Protein structures were visualized in PyMOL (77). Interaction interfaces were inferred using the PISA server (78).

Crystal structure determination and refinement

During the purification of IL-18BP, purified IL-18 was added in a 1:2 ratio prior to caspase 3 and Endo H digests. The complex was purified via SEC and concentrated to 35 mg ml⁻¹. Sitting-drop vapor diffusion experiments were set up in Swissci 96-well triple drop plates (Molecular dimensions) with commercial sparse matrix crystallization screens. Using a Mosquito liquid handling robot (TTP Labtech), the protein was mixed with mother liquor in a 1:1 ratio in a final volume of 200 nl. The plates were incubated at 20 °C. An initial hit in the Crystal Screen (Cat No HR2-110, Hampton Research) in condition D10 (0.2 M calcium acetate hydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, 18% w/v polyethylene glycol 8000) was optimized by varying pH (6.2–7.1) and PEG concentration (12%–27%). Crystals obtained from the optimization screen were used for seeding new plates, finally yielding single crystals. These crystals were cryoprotected in mother liquor supplemented with 20% glucose prior to cryocooling in liquid nitrogen. Diffraction data was initially collected under cryogenic conditions at a beamline P14 of synchrotron PETRA III (Hamburg, Germany).

The diffraction data were integrated using XDS (79) and were treated for anisotropy using the STARANISO server (55). Initial phases were determined by maximum-likelihood molecular replacement in Phaser (80) using a search model derived from the structure of IL-18 in complex with ectvIL-18BP (pdb 3f62). Iterative cycles of structure building and refinement were performed in COOT (81) and Phenix.refine (82), respectively.

Structure coordinates and structure factors for the human IL-18:IL18BP complex have been deposited in the Protein Data Bank (PDB) under accession code 7al7.

Data availability

Structure coordinates and structure factors for the human IL-18:IL18BP complex have been deposited in the Protein Data Bank (PDB) under accession code 7al7.

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Abbreviations—The abbreviations used are: AOSD, Adult-onset Still’s disease; CD, Crohn’s disease; COVID-19, Coronavirus disease; ectvIL-18BP, ectromelia poxvirus IL-18-binding protein; Endo H, Endoglycosidase H; HBS, Hepes-buffered saline; IFN-γ, interferon-γ; IL-18, interleukin-18; MALLS, multi-angle laser light scattering; RA, rheumatoid arthritis; SEC, size-exclusion chromatography; yldvIL-18BP, yaba-like disease virus disease IL-18-binding protein.

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