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Full-length and N-terminally truncated recombinant interleukin-38 variants are similarly inefficient in antagonizing interleukin-36 and interleukin-1 receptors

Alejandro Diaz-Barreiro[1,2,3], Dominique Talabot-Ayer[1,2,3], Arnaud Huard[1,2,3], Gea Cereghetti[4], Jenna Tonacini[1,2,3], Mike Maillasson[5,6], Antonio Francés-Monerris[7], Erwan Mortier[5,6] and Gaby Palmer[1,2,3*]

Abstract

Background Interleukin (IL)-38 is an IL-1 family cytokine that was proposed to exert anti-inflammatory effects. However, its mechanisms of action are not well understood and the identity of the IL-38 receptor(s) remains debated. Proposed candidates include the IL-1 receptor (IL-1R1), the IL-36 receptor (IL-36R) and the orphan receptor IL-1RAPL1. Yet, in literature, IL-38 is often presented as an IL-36R antagonist.

Methods The N-terminus of the IL-38 protein produced in a human keratinocyte cell line and of endogenous epidermal IL-38 isolated from healthy human skin was characterized by mass spectrometry. The effects of various recombinant forms of IL-38 on IL-36R- and IL-1R1-mediated responses were assessed in IL-36R HEK Blue reporter cells and in a normal human keratinocyte cell line. IL-8 and IL-6 production was quantified by ELISA. Binding of recombinant IL-38 proteins to the IL-36R was assessed by surface plasmon resonance.

Results Analysis of its native N-terminus revealed that the IL-38 protein produced by human keratinocytes starts at cysteine 2. In cell-based assays, neither full-length amino acid 2-152 IL-38 nor two N-terminally truncated forms of the protein showed efficient antagonist activity on IL-36R- and IL-1R1-mediated responses. The recombinant IL-38 proteins bound to the IL-36R with only moderate affinity, which may provide a mechanistic explanation for inefficient IL-36R antagonism.

Conclusions Our results argue against meaningful inhibitory effects of any of the recombinant IL-38 variants tested on IL-36R or IL-1R1-mediated responses. The mechanisms underlying reported anti-inflammatory effects of IL-38 are thus still unclear, but seem unlikely to be mediated by classical IL-36R or IL-1R1 antagonism.

Keywords Cytokine, Interleukin-1 family, Interleukin-38, Receptor antagonist

*Correspondence:
Gaby Palmer
gaby.palmer@unige.ch
[1]Division of Rheumatology, Department of Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland
[2]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1 rue Michel-Servet, Geneva CH-1211, Switzerland
[3]Geneva Centre for Inflammation Research, Geneva, Switzerland
[4]Department of Chemistry, University of Cambridge, Cambridge, UK
[5]Nantes Université, CNRS, Inserm, CRCI 2 NA, Nantes, France
[6]Nantes Université, Centre Hospitalo-Universitaire (CHU) Nantes, Inserm, CNRS, SFR Bonamy, UMS BioCore, Imp@ct Platform, Inserm, Nantes, France
[7]Institut de Ciència Molecular, Universitat de València, P.O. Box 22085, València 46071, Spain

Background

Cytokines of the interleukin (IL)-1 family play important roles in the initiation and regulation of inflammatory responses. The family includes 11 members, of which 7 are pro-inflammatory agonists (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and 4 are described as anti-inflammatory cytokines (IL-1Ra, IL-36Ra, IL-37, IL-38). The pro-inflammatory IL-1 family members bind to specific cell-surface receptors belonging to the IL-1 receptor (IL-1R) family. In particular, IL-1α and IL-1β bind to IL-1R1, while IL-36α, IL-36β, and IL-36γ bind to the IL-36 receptor (IL-36R). This leads to the recruitment of a second receptor chain, the IL-1R accessory protein (IL-1RAP) and to activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling, resulting in the expression of inflammatory genes [1]. The naturally occurring receptor antagonists, IL-1Ra and IL-36Ra, competitively inhibit the pro-inflammatory activity of their cognate agonists by binding with high affinity to, respectively, IL-1R1 and IL-36R, without allowing for the recruitment of IL-1RAP. IL-1Ra thus inhibits the IL-1R1-mediated activity of IL-1α and IL1β [2], and IL-36Ra blocks IL-36R-mediated effects of IL-36α, IL-36β, and IL-36γ [3]. This receptor antagonism is dose dependent and complete inhibition typically requires a 100-fold excess of receptor antagonist over the amount of agonist. The interaction of the receptor antagonists with their respective receptors is highly specific, so that IL-36Ra does not decrease IL-1 activity and IL-1Ra does not inhibit the effect of IL-36 agonists, even at high concentrations [4].

IL-38 shares over 40% amino acid (aa) sequence homology with IL-1Ra and IL-36Ra [5]. However, although anti-inflammatory effects have also been reported for IL-38 [6], its mechanisms of action are not well understood. IL-38 was first proposed to bind IL-1R1, although with lower affinity than IL-1Ra or IL-1β [7]. Later, IL-38 was reported to bind IL-36R, rather than IL-1R1, and to act as an IL-36R antagonist [8]. In addition, the orphan receptor interleukin-1 receptor accessory protein-like 1 (IL-1RAPL1) was also proposed to mediate effects of IL-38 [9]. At this stage, the identity of the IL-38 receptor(s) thus remains debated. Nevertheless, in the literature, IL-38 is often presented as an IL-36R antagonist.

The activity of several IL-1 family cytokines is enhanced by proteolysis, which results in the generation of N-terminally truncated, so-called 'mature' forms of the proteins [10]. This led to the suggestion that IL-38 might also require proteolytic activation for optimal bioactivity [9]. In silico analyses predicted cleavage sites for proteases such as calpain, cathepsin G, and granzyme B, which have been shown to proteolytically activate other IL-1 family cytokines [11–14], in the N-terminus of human IL-38 [15]. Additional potential cleavage sites were identified for matrix metalloproteinase (MMP)-2 and MMP 9 [15]. The predicted MMP-2 site is located between aa 19 and 20 of human IL-38, nine amino acids N-terminal to an A-x-Asp motif, in which A represents an aliphatic amino acid. Cleavage at this particular distance N-terminally of an A-x-Asp sequence was previously shown to enhance the biological activity of other IL-1 family members [3] and this 'A-x-Asp rule' was used as a rationale for the generation of a truncated aa20-152 recombinant form of IL-38 [9]. However, to date, neither specific cleavage site(s), nor protease(s) involved in IL-38 truncation have been identified experimentally and, while several recombinant IL-38 proteins with different N-terminal truncations have been used in different studies [8, 9, 16–21], a consensus identifying a unique, optimally bioactive, truncated IL-38 variant has not yet emerged [6]. In this study, we determined the N-terminal sequence of the native IL-38 protein produced by a human keratinocyte cell line, and of endogenous IL-38 isolated from healthy human epidermis. Then, in an effort to systematically investigate the effects of different recombinant forms of IL-38 on specific, molecularly defined responses, we studied the effects of this full-length form of IL-38 and of shorter, N-terminally truncated IL-38 variants on IL-36R- and IL-1R1-mediated readouts in well-controlled experimental systems and evaluated their affinity for the IL-36R. Taken together, our observations argue against a biologically meaningful role for the tested IL-38 proteins as IL-36R or IL-1R1 antagonists.

Methods

Recombinant cytokines

Recombinant human IL-36γ (cat n°6835-IL), human IL-1α (cat n°200-LA), human secreted (s)IL-1Ra (cat n°280-RA) and human IL-36Ra (cat n°1275-IL) were purchased from R&D Systems (Abingdon, UK) and resuspended at 10 (IL-1α), 100 (IL-36γ, sIL-1Ra), or 125 (IL-36Ra) µg/ml in PBS (IL-36Ra), PBS containing 0.1% BSA (IL-1α, sIL-1Ra) or H_2O (IL-36γ), as recommended by the manufacturer. Recombinant C-terminally His-tagged human aa2-152 IL-38 (cat n°AG-40 A-0191), aa20-152 IL-38:Fc-knobs-in-holes (KIH) linked human monomeric IL-38 (cat n°AG-40B-0226), and the corresponding human Fc-KIH IgG1 negative control (cat n° AG-35B-0015) were purchased from Adipogen Life Sciences (Fuellinsdorf, Switzerland) and resuspended at 100 (aa20-152 IL-38:Fc-KIH, Fc-KIH IgG1) or 500 (2-152 His-tagged) µg/ml in H_20, as recommended by the manufacturer. Endotoxin content indicated by the manufacturer was < 0.10 EU/µg protein for sIL-1Ra and IL-36Ra, <1EU/µg protein for His-tagged human aa2-152 IL-38, and < 0.01EU/µg protein for aa20-152 IL-38:Fc and Fc-KIH IgG1. Recombinant human aa2-152, aa3-152 IL-38 and intracellular IL-1Ra (icIL-1Ra1) [22] were made in house. The corresponding human IL-38 and icIL-1Ra1 cDNA

sequences were codon optimized for E. coli, synthetized and cloned by GenScript Biotech into a pET21a vector (Addgene, Watertown, MA, USA) in order to add a TEV protease cleavable N-terminal 6xHis tag. Proteins were expressed in LOBSTR BL21 (DE3) E. coli cells (EC1002, Kerafast, Shirley, MA, USA) and pellets were lyzed in 50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 5% glycerol, 0.5% Triton X-100, 7.5 mM β-mercaptoethanol (β-ME), supplemented with an anti-protease cocktail. High β-mercaptoethanol concentrations were maintained during the whole purification procedure in order to ensure reducing conditions. Affinity purification was carried out on a His-trap FF column (17525501, Cytiva, Marlborough, MA, USA) and the N-terminal tag was cleaved with His-TEV protease during dialysis against 20 mM Tris pH 8; 250 mM NaCl; 5% glycerol, 15 mM β-ME. Then, proteins of interest were separated from contaminants (uncleaved protein, TEV protease, tag) on a His-trap FF column, and purified on a Q-HP column (17115401, Cytiva). Fractions were analyzed by SDS-PAGE, IL-38 containing fractions were pooled, concentrated on 10 kDa MW cutoff Amicon concentrators (UFC901024, Sigma-Aldrich, Saint Louis, MO, USA) and subjected to size exclusion chromatography (SEC) on a Superdex 75, 10/300 column (29148721, Cytiva) in sterile 30 mM Hepes pH 7, 500mM NaCl, 5% glycerol, 1 mM β-ME. Fractions were analyzed by SDS-PAGE and fractions containing pure proteins were pooled, flash-frozen in liquid nitrogen and stored as 1 mg/ml stock solutions at -80 °C. Endotoxin content of recombinant cytokines produced in house was less than 0.01 EU/µg of protein, as determined in 2 independent purifications using a Limulus Amoebocyte Lysate (LAL) assay (Pierce™ Chromogenic Endotoxin Quant Kit, Thermo Fisher Scientific, Waltham, MA, USA). The sequences, and in particular the correct N-termini, of recombinant C-terminally His-tagged aa2-152 IL-38 (Adipogen) and of recombinant aa2-152 and 3-152 IL-38 produced in house were verified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (Table S1). The molecular weights of all recombinant proteins, as well as their concentrations used in cell-based assays, expressed in ng/ml and nmol/L (nM), are listed in Table 1.

Table 1 Molecular weights and concentrations of recombinant proteins

recombinant protein	MW (kDa)	concentration	equivalent concentration
		(ng/ml)	(nM)
aa2-152His IL-38	18	10	0.6
		100	5.6
		1000	55.6
aa2-152 IL-38	17	10	0.6
		100	5.9
		1000	58.8
aa3-152 IL-38	17	10	0.6
		100	5.9
		1000	58.8
aa20-152 IL-38:Fc-KIH	78	30	0.4
		300	3.8
		3000	38.5
IL-36Ra	17	10	0.6
		100	5.9
		1000	58.8
sIL-1Ra	17	1	0.06
		10	0.6
		100	5.9
		1000	58.8
icIL-1Ra1	18	1	0.06
		10	0.6
		100	5.6
IL-36g	17	0.1	0.006
		10	0.6
IL-1a	18	1	0.06
Fc-KIH IgG1	28	17	0.6
		170	6.1
		1700	60.7

The molecular weights (MW) of the recombinant proteins included in this study, and their concentrations used in cell-based assays, expressed in ng/ml and nmol/L (nM), are indicated

Cell culture and stimulation

Human embryonic kidney (HEK) 293T cells and IL-36R HEK Blue reporter cells (InvivoGen, Toulouse, France) were cultured in DMEM (4.5 g/l glucose) supplemented with L-glutamine, streptomycin, penicillin and 10% FCS, at 37 °C in a humidified atmosphere containing 5% CO2. For IL-36γ and IL-1α stimulation assays, IL-36R HEK Blue cells were seeded in 96-well plates at a density of 100'000 cells/ml, corresponding to 20'000 cells/well. On the next day, cells were preincubated with recombinant IL-38, IL-36Ra or IL-1Ra proteins (1-1000 ng/ml, corresponding to 0.06–58.8 nM, resp. 30-3000 ng/ml, corresponding to 0.4–38.5 nM, for aa20-152 IL-38:Fc-KIH) and stimulated 15 min later with IL-36γ or IL-1α at indicated concentrations. Higher ng/ml concentrations were used for aa20-152 IL-38:Fc-KIH, as compared to other IL-38 variants, in order to adjust for the molecular weight of this fusion protein (78 kDa), which is substantially higher than that of IL-38 alone (17 kDa) (Table 1). For practical reasons, we nevertheless capped the amount of aa20-152 IL-38:Fc at 3000 ng/ml (38.5 nM), which corresponds to 0.7 molar equivalents of 1000 ng/ml aa2-152His, aa2-152 or aa3-152 IL-38 (55.6–58.8 nM). Culture supernatants were harvested 6 h later to measure secreted embryonic alkaline phosphatase (SEAP) activity or 24 h later to assess IL-8 levels by ELISA. For flow cytometry, HEK and IL-36R HEK Blue cells were seeded in T25 cell culture flasks at a density of 200'000 cells/ml, corresponding to 1×10^6 cells/flask. On the next day, cells

were detached with 0.05% trypsin, 0.02% EDTA at 37 °C for 5 min.

Immortalized Normal Human Keratinocyte (NHK) cells [23] and IL-38 expressing NHK/38 cells [24] were cultured in keratinocyte-serum free medium (K-SFM, Thermo Fisher Scientific), supplemented with bovine pituitary extract and human recombinant epidermal growth factor (Thermo Fisher Scientific), penicillin and streptomycin, at 37 °C in a humidified atmosphere containing 5% CO2. The generation of NHK/38 cells, which allow for doxycycline (Dox)-inducible IL-38 overexpression using the T-REx system (Thermo Fisher Scientific), was described in detail previously [24]. Briefly, NHK cells were first transfected with the pcDNA6/TR vector, which encodes the tetracycline repressor (TR) protein. Antibiotic selection and limiting dilution cloning were then used to isolate a clone expressing high levels of TR. This clone was further transfected with plasmid pcDNA4/TO/hIL-38, containing the human full-length IL-38 coding sequence (GenBank accession n° NM_173161.3, bp 76–534) placed under the control of a TR-regulated promoter, to obtain stably transfected NHK/38 cells. To identify the N-terminus of the IL-38 protein produced by human keratinocytes, NHK/38 cells were seeded in 6-well plates at a density of 400'000 cells/well. On the next day, cells were treated with 1 µg/ml Dox for 24 h to induce IL-38 expression. For IL-36γ and IL-1α stimulation assays, NHK cells were seeded in 96-well plates at a density of 100'000 cells/ml, corresponding to 20'000 cells/well. On the next day, cells were preincubated with recombinant IL-38, IL-36Ra or IL-1Ra proteins as indicated and stimulated 15 min later with IL-36γ or IL-1α at indicated concentrations. Culture supernatants were harvested 24 h later and IL-8 or IL-6 levels were assessed by ELISA. For flow cytometry, NHK cells were seeded in T25 cell culture flasks at a density of 200'000 cells/ml, corresponding to 1×10^6 cells/flask. On the next day, the cells were treated with Versene for 2 min and detached with 0.05% trypsin, 0.02% EDTA at 37 °C for 5 min.

Human skin samples

Skin biopsies were taken from healthy adults undergoing surgery at the Department of Plastic and Reconstructive Surgery of the Geneva University Hospitals in Switzerland. Whole skin was digested with dispase (5 U/ml, STEMCELL Technologies, Vancouver, Canada) overnight at 4 °C to detach the epidermis from the dermis, as previously described (Talabot-Ayer et al., 2019). The epidermal layer was collected and frozen.

Analysis of IL-38 protein N-terminal sequences

The N-terminal sequences of recombinant C-terminally His-tagged aa2-152 IL-38 (Adipogen), recombinant aa2-152 and aa3-152 IL-38 prepared in house, the IL-38 protein produced by NHK/38 cells, and the endogenous IL-38 protein isolated from healthy human epidermis were verified by LC-ESI-MS/MS. Dox-treated NHK/38 cells were lyzed in TNT buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100) supplemented with protease inhibitors. 40–100 mg of human epidermis were lyzed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche) and 40 mM iodoacetamide, using a Precellys tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) with Tissue grinding CKMix50-R tubes and the following program: 3 cycles of 20 s at 8000 rpm, each followed by a 30 s rest period. Samples were incubated for 30 min on ice before clearing. IL-38 was immunoprecipitated from NHK/38 cell and epidermal lysates using a polyclonal goat anti-IL38 antibody (AF2427, R&D Systems). The specificity of the immunoprecipitation was verified by using normal goat IgG (005-000-003, Jackson Immuno Research Europe Ltd) as a negative control. Recombinant IL-38 proteins and IL-38 immunoprecipitated from NHK/38 cells and from human epidermis were separated electrophoretically on a 4–12% gradient reducing SDS-PAGE (NHK/38 cells; NuPAGE; Thermo Fisher Scientific) or on a mPAGE 4–20% gradient Bis-Tris SDS-PAGE (human epidermis; Merck), stained with Coomassie blue (Thermo Fisher Scientific) or silver stain (Thermo Fisher Scientific), or transferred onto PVDF membranes for Western blotting. The membranes were blocked with 5% horse serum in TBS, 0.05% Triton X-100 (TBST) and probed with a biotinylated polyclonal goat anti-IL38 antibody (BAF2427, R&D Systems, 1/1000) in TBST. Immunoreactive bands were detected with streptavidin HRP (BD Biosciences, San Jose, CA, 1/3000) and Radiance Plus chemiluminescent detection (Azure Biosystems, Dublin, CA) on a LAS4000 imager (Fujifilm Life Science, Düsseldorf, Germany). For LC-ESI-MS/MS, the bands migrating at the expected size for IL-38 (17 kD) were excised from Coomassie blue stained gels (Figure S1) and the proteins contained therein were reduced, alkylated and in-gel digested using either Lys-C or trypsin. Peptides were analyzed by LC-ESI-MS/MS using an Easy nLC 1000 liquid chromatography system (Thermo Fisher Scientific), coupled with a Q-Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). Database searches were performed with Mascot (Matrix Science) using the Human Proteome Reference database (Uniprot), combined with an in-house database containing truncated N-terminal sequences of IL-38 without and with His tag, and considering cysteine carbamidomethylation, asparagine and glutamine deamidation, methionine oxidation and N-terminal acetylation as post-translational modifications. Data were analyzed and validated with Scaffold (Proteome Software). Protein identifications were accepted if they could be established

at greater than 95.0% probability and contained at least 2 identified peptides. The sequences and mass spectrometric characteristics of all peptides identified for the IL-38 protein produced by NHK/38 cells, for endogenous IL-38 isolated from healthy human epidermis, as well as for recombinant aa2-152His, aa2-152 and aa3-152 IL-38 are listed in Tables 1, 2, 3, 4 and 5 of Table S1.

Analysis of recombinant cytokine purity

Commercial and in-house produced recombinant cytokines (1 µg) were separated electrophoretically on a 4–12% gradient reducing SDS-PAGE (NuPAGE; Thermo Fisher Scientific) and stained with Coomassie Blue (Thermo Fisher Scientific) or One-Step Blue (Biotium Inc., Freemont, CA) to evaluate their purity.

Circular dichroism

Circular Dichroism (CD) spectra were recorded on a J-810 CD Spectrometer (Jasco, Easton, MD) with a Peltier temperature-controlled cell holder using a quartz cuvette with a 1 mm path length (Hellma Analytics, Southend on Sea, UK) and analyzed using BeStSel [25, 26]. Recombinant aa2-152 IL-38 was thawed on ice, centrifuged for 10 min at 4 °C at 21'000 g, and diluted to a concentration of 0.5 mg/ml prior to measurement. All measurements were performed at 22 °C.

Molecular graphics

Molecular graphics were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311.

ELISA

IL-8 and IL-6 levels in culture supernatants were assessed by ELISA (Thermo Fisher Scientific) following the manufacturer's instructions.

Flow cytometry

For flow cytometry, HEK, IL-36R HEK Blue and NHK cells were pelleted by centrifugation and blocked with Fc receptor blocking reagent (BD Biosciences, Allschwil, Switzerland) in PBS, 0.5% BSA for 10 min. Cells were stained with a biotinylated polyclonal goat anti-IL-36R antibody (BAF872, R&D Systems; dilution 1/200) or with a PE-conjugated anti-IL-1R1 antibody (FAB269P, R&D Systems; dilution 1/10) for 25 min, or left unstained as a negative control. Cells were then washed and anti-IL-36R-labeled cells were further incubated with streptavidin-PE (12-4317-87, Thermo Fisher Scientific; dilution 1/100) for 15 min. Samples were acquired on the BD LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo V10 (BD Biosciences).

Secreted embryonic alkaline phosphatase assay

Secreted embryonic alkaline phosphatase (SEAP) activity in culture supernatants was assessed using the QUANTI-Blue™ solution (InvivoGen) according to the manufacturer's instructions.

Surface plasmon resonance studies

Surface plasmon resonance (SPR) experiments were performed with a BIAcore T200 biosensor (BIAcore, Uppsala, Sweden). Monoclonal anti-human IgG (Fc) antibodies were covalently linked to CM5 sensor chips by amine coupling and used to capture a recombinant human IL-36R-Fc chimeric protein (200 nM, 872-RP, R&D Systems). The binding of increasing concentrations of recombinant human IL-36Ra (62.5–1000 nM), used as a positive control, IL-38 variants (aa 2-152 with C-terminal His tag, 375–6000 nM; aa 2-152, 375–6000 nM; aa3-152, 375–6000 nM), and icIL-1Ra1 (375–6000 nM), used as a negative control, was monitored at 25 °C in HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) running buffer (Cytiva, Versailles, France) using single cycle kinetics with an association time of 120 s, a dissociation time of 600 s and a flow rate of 40 µL/min. Analysis of sensorgrams was performed using the BIAeval two state reaction model.

Statistical analyses

Data were analyzed with Prism version 8 (Graphpad Software, La Jolla, USA) using one-way ANOVA followed by Dunnett's multiple comparisons test or two-way ANOVA followed by Sidak's multiple comparisons test, as indicated. Values are expressed as mean ± SEM. Statistical significance was defined at a p value < 0.05.

Results

IL-38 starts at cysteine 2 in an IL-38 overexpressing human keratinocyte cell line and in normal human epidermis

N-terminal truncation, which is observed for instance in apoptotic cells [9], was proposed to promote IL-38 bioactivity. However, the native N-terminal sequence of IL-38 produced by healthy human cells has not been characterized. IL-38 is constitutively produced in keratinocytes in human skin [24]. We therefore immunoprecipitated the native IL-38 protein produced by an IL-38 overexpressing human keratinocyte cell line [24] (Fig. 1A, Figure S1) and by primary keratinocytes in healthy human epidermis (Fig. 1B) to analyze its N-terminal sequence by LC-ESI-MS/MS. A limited number of peptide-spectrum matches (PSMs) was obtained, probably due to a low initial amount of protein in the immunoprecipitates. Nevertheless, IL-38 was clearly identified with 13 PSMs from NHK/38 cells, and 8 PSMs from human epidermis, corresponding respectively to 3 and 2 exclusive unique peptide sequences. In both cases, the detected N-terminal

sequence was CSLPMARYYIIK, matching the full human IL-38 sequence minus the N-terminal initiator methionine (Fig. 1C, Table S1). Our analysis further indicated that the N-terminal cysteine was acetylated (Table S1). Taken together, these data strongly suggest that the native IL-38 protein produced by NHK/38 cells, as well as by keratinocytes in healthy skin in vivo, undergoes N-terminal methionine excision and thus starts at cysteine 2.

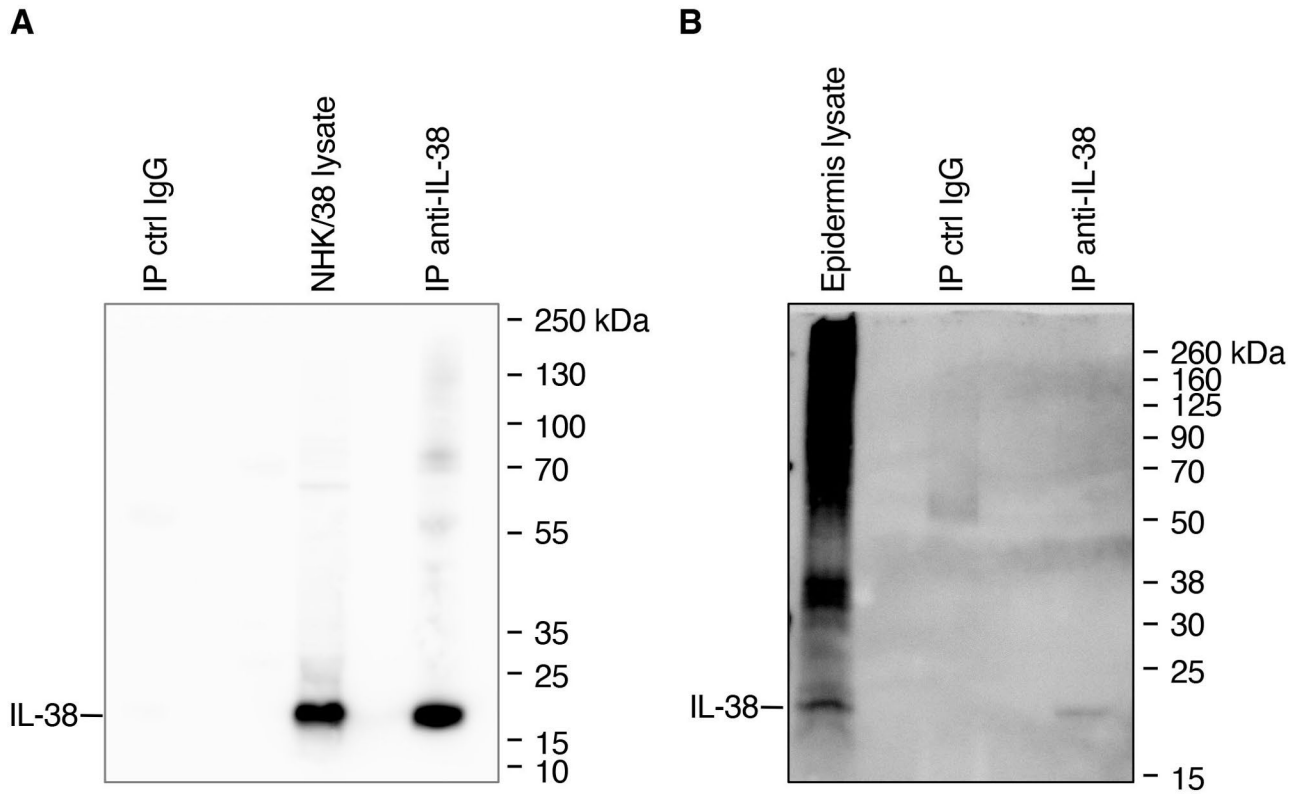
Recombinant IL-38 proteins do not inhibit IL-36 activity in IL-36R reporter cells

Based on this information, we investigated a potential IL-36 receptor (IL-36R) antagonistic effect of aa2-152 IL-38. We used 2 different preparations of aa2-152 recombinant human IL-38 proteins, namely a commercial C-terminally His tagged aa 2-152 protein, and untagged, in house-produced aa2-152 IL-38. In addition, we also used an untagged, in house-produced aa3-152 IL-38 variant and a commercial aa20-152 IL-38:Fc-KIH fusion protein, based on previously reported [9, 18] improved biological activities of these truncated forms over the full length recombinant IL-38 protein. We first evaluated the purity of the IL-38 protein preparations, as well as of related proteins used in the activity assays described below, by SDS-PAGE and Coomassie Blue staining (Fig. 2A). All samples revealed a major band of expected size, with no, or negligible, evidence of unexpected additional bands. We further confirmed proper folding of the recombinant aa2-152 IL-38 protein by analyzing its circular dichroism (CD) spectrum, which revealed a high content of β-sheets (Fig. 2B), similar to other family members [27–29]. Software analysis [25, 26] estimated the percentage of β-strands at approximately 40%, consistent with the 40.1% expected from the IL-38 crystal structure (Fig. 2C, β-strands in orange; PDB: 5BOW; https://www.rcsb.org/structure/5bow), which exhibits the characteristic 12 β-trefoil fold of IL-1 family cytokines [30].

Next, we assessed the effects of these proteins on IL-36 receptor (IL-36R)-mediated responses using the commercial IL-36R HEK Blue reporter cell line (Fig. 3A). To maximize the chances that even weak antagonistic effects would be detected, we stimulated IL-36R HEK Blue cells with the lowest IL-36γ concentration at which we could see robust induction of the SEAP reporter, which was 0.1 ng/ml, corresponding to 0.006 nM (Figure S2A), in agreement with information provided by the manufacturer. Unfortunately, SEAP reporter levels proved unstable over passages in this cell line, with variable and often very high background at baseline (data not shown). We thus preferentially used IL-8 secretion, which was robustly and consistently induced by IL-36γ in all experiments, as our primary readout (Fig. 3A). IL-36 receptor antagonist (IL-36Ra) served as a positive control to inhibit IL-36R activation by IL-36γ. The effect of 0.1 ng/ml (0.006 nM) IL-36γ was partly reduced by IL-36Ra at 10 ng/ml (0.6 nM), thus at a 100-fold molar excess, and completely inhibited at 100 ng/ml (5.9 nM), which represents a 1000-fold molar excess. In contrast, none of the IL-38 proteins tested had any significant effect on IL-36γ-induced IL-8 production at concentrations ranging from 10 to 1000 ng/ml (0.6–58.8 nM) for the different IL-38 variants, or from 30 to 3000 ng/ml (0.4–38.5 nM) for the aa20-152 IL-38:Fc-KIH fusion protein, which represents a 70 to 10'000-fold molar excess as compared to IL-36γ for all proteins tested. Similarly, as expected, IL-1 receptor antagonist (IL-1Ra), used as a negative control, had no effect on this IL-36R-mediated response. Unfused Fc-KIH IgG1, which was used as an additional specificity control for the aa20-152 IL-38:Fc-KIH fusion protein, marginally reduced IL-36γ-induced IL-8 production in a dose-independent manner, suggesting a non-specific effect (Figure S3A). Of note, none of the recombinant proteins tested affected SEAP or IL-8 production in IL-36R HEK Blue cells at baseline, in absence of IL-36γ (Figure S4A). Altogether, these cell-based assays indicate that, in contrast to IL-36Ra, the different IL-38 variants tested do not efficiently antagonize IL-36γ activity in the IL-36R HEK Blue reporter cell line.

Recombinant IL-38 proteins do not inhibit IL-36 activity in human keratinocytes

IL-36R HEK Blue cells represent an artificial system, in which the IL-36R is strongly overexpressed. To verify our observations in a cell type that naturally responds to IL-36 [16, 31], we used the human NHK keratinocyte cell line [23]. We observed robust induction of IL-8 secretion in response to 10 ng/ml (0.6 nM) IL-36γ (Figure S2B). The differences in sensitivity of IL-36R overexpressing HEK Blue cells and NHK cells to IL-36γ are consistent with their respective cell surface levels of IL-36R (Figure S2C). In NHK cells, the effect of 10 ng/ml (0.6 nM) IL-36γ was partially blocked by 100 ng/ml (5.9 nM) IL-36Ra, thus at a 10-fold molar excess, and completely inhibited at 1000 ng/ml (58.8 nM), which corresponds to a 100-fold molar excess of IL-36Ra over IL-36γ (Fig. 3B). The IL-36Ra/IL-36γ molar excess required to block IL-36R receptor activity was thus 10 times lower in NHK than in IL-36R HEK Blue cells. Again, this likely reflects the difference in IL-36R expression levels between the two cell types, as high IL-36R receptor levels on HEK Blue cells are expected to make efficient inhibition of all available receptor molecules by the antagonist more difficult. However, despite a more favorable situation in NHK cells, none of the IL-38 proteins tested displayed any significant effect on IL-36γ-induced IL-8 production at concentrations corresponding to a 0.7 to 100-fold molar excess as compared to IL-36γ for all proteins tested



C

NHK/38 cells

IL1FA_HUMAN (100%), 16 943,6 Da
Interleukin-1 family member 10 OS=Homo sapiens OX=9606 GN=IL1F10 PE=1 SV=1
3 exclusive unique peptides, 9 exclusive unique spectra, 13 total spectra, 48/152 amino acids (32% coverage)

M C S L P M A R Y Y I I K Y A D Q K A L Y T R D G Q L L V G D P V A D N C C A E
K I C I L P N R G L A R T K V P I F L G I Q G G S R C L A C V E T E E G P S L Q
L E D V N I E E L Y K G G E E A T R F T F F Q S S S G S A F R L E A A A W P G W
F L C G P A E P Q Q P V Q L T K E S E P S A R T K F Y F E Q S W

Normal human epidermis

IL1FA_HUMAN (100%), 16 943,6 Da
Interleukin-1 family member 10 OS=Homo sapiens OX=9606 GN=IL1F10 PE=1 SV=1
2 exclusive unique peptides, 3 exclusive unique spectra, 8 total spectra, 35/152 amino acids (23% coverage)

M C S L P M A R Y Y I I K Y A D Q K A L Y T R D G Q L L V G D P V A D N C C A E
K I C I L P N R G L A R T K V P I F L G I Q G G S R C L A C V E T E E G P S L Q
L E D V N I E E L Y K G G E E A T R F T F F Q S S S G S A F R L E A A A W P G W
F L C G P A E P Q Q P V Q L T K E S E P S A R T K F Y F E Q S W

Fig. 1 (See legend on next page.)

(Fig. 3B). Similarly, as expected, IL-1Ra (Fig. 3B) and Fc-KIH IgG1 (Figure S3B) had no effect on this IL-36R-dependent readout.

A previous study reported an inhibitory effect of IL-38 on several IL-36γ-induced responses, including *IL6* mRNA expression, at 0.1 to 10-fold IL-38/IL-36γ molar ratios in keratinocytes [16]. We thus wondered whether

(See figure on previous page.)

Fig. 1 Proteolytic coverage map of human IL-38 immunoprecipitated from NHK/38 cells and from normal epidermis. **A**. NHK/38 cells were lyzed and immunoprecipitated with a polyclonal goat anti-IL-38 antibody (IP anti-IL-38, right lane). Normal goat IgG was used as a negative control to assess the specificity of the immunoprecipitation (IP ctrl IgG, left lane). NHK/38 whole-cell lysates (middle lane) and immunoprecipitated proteins were fractionated by SDS-PAGE and IL-38 was detected by Western blotting. The results are representative of $n = 6$ independent experiments. **B**. Normal human epidermis was lyzed and immunoprecipitated with a polyclonal goat anti-IL-38 antibody (IP anti-IL-38, right lane). Normal goat IgG was used as a negative control to assess the specificity of the immunoprecipitation (IP ctrl IgG, middle lane). Epidermis whole-cell lysates (left lane) and immunoprecipitated proteins were fractionated by SDS-PAGE and IL-38 was detected by Western blotting. The results are representative of $n = 2$ independent experiments. **C**. The amino acid sequence of human IL-38 is shown. Peptides identified by LC-ESI-MS/MS analysis of IL-38 immunoprecipitated from IL-38 expressing NHK cells (NHK/38; top panel) and normal human epidermis (bottom panel) lysates are highlighted in yellow. The identified peptides cover 32% of the NHK/38 IL-38 protein (48/151 aa, top panel) and 23% of endogenous epidermal IL-38 protein (35/152 aa, bottom panel). The detected N-terminal sequence was CSLPMARYYIIK, corresponding to the IL-38 sequence minus the initiator methionine and thus starting at cysteine 2. Post-translationally modified residues (Table S1), including the acetylated N-terminal cysteine, are highlighted in green

the observed lack of effect of IL-38 observed in the present study might be readout specific. Therefore, we analyzed IL-6 production as a second readout in IL-36γ-stimulated NHK cells (Figure S2D). However, as for IL-8 secretion, while IL-36Ra efficiently blocked IL-36γ-induced IL-6 production, IL-38 and IL-1Ra did not.

Taken together, our observations indicate that, in contrast to IL-36Ra, the various IL-38 proteins tested do not affect two different IL-36γ-induced readouts in human keratinocytes that naturally express IL-36R. Finally, none of the recombinants tested affected IL-8 or IL-6 production in NHK cells at baseline, in absence of IL-36γ (Figure S4B and data not shown).

Recombinant IL-38 proteins bind to the IL-36R only with moderate affinity

The negative results obtained with the different IL-38 variants in cell-based assays prompted us to directly investigate their binding to an immobilized IL-36R-Fc chimeric protein by SPR (Table 2). The aa2-152 form of IL-38, with or without a C-terminal His tag, bound to the IL-36R only with moderate affinity, as illustrated by a KD of around 300 nM. The affinity measured for aa3-152 IL-38 was even lower, with a KD in the μmolar range. IL-36Ra binds to the IL-36R with higher affinity than the three IL-38 proteins tested. We observed a KD of 48 nM for this interaction, consistent with previously published observations [3, 32, 33]. In contrast, as expected, icIL-1Ra1, used as a negative control, exhibited extremely low affinity for the IL-36R. Finally, binding of the aa20-152 IL-38:Fc-KIH fusion protein could unfortunately not be evaluated in this assay, in which we used Fc capture for receptor immobilization, as this IL-38 variant carries an Fc moiety itself. Altogether, our observations highlight moderate to low affinity of the different IL-38 variants tested for the IL-36R.

Recombinant IL-38 proteins do not efficiently inhibit IL-1 activity in NHK and HEK cells

In addition to acting as an IL-36R antagonist, IL-38 was also proposed to decrease IL-1 responses [7, 9, 34]. We thus investigated the effects of the different recombinant IL-38 proteins on IL-1α–induced IL-8 production in NHK cells (Fig. 4A). IL-1α efficiently induced IL-8 secretion at 1 ng/ml (0.06 nM) in these cells (Figure S5A), consistent with cell-surface expression of IL-1R1 (Figure S5B). The effect of 1 ng/ml (0.06 nM) IL-1α on IL-8 secretion was partially blocked by 1 ng/ml (0.06 nM) sIL-1Ra, thus at a 1:1 molar ratio, and completely inhibited by 10 ng/ml (0.6 nM) sIL-1Ra, corresponding to a 10-fold molar excess (Fig. 4A).

IL-1Ra exists as different isoforms, derived from the same gene through the use of alternative start sites and splicing. Expression of the different isoforms is cell-type specific [35]. The secreted (s)IL-1Ra isoform, which is released in particular by myeloid cells, is commercially available. In addition, we produced a recombinant protein corresponding to the intracellular (ic)IL-1Ra1 isoform, which is the IL-1Ra isoform that is naturally produced by keratinocytes and other epithelial cells [22]. Like sIL-1Ra, recombinant human icIL-1Ra1 efficiently blocked IL-1α–induced IL-8 secretion, with a partial, but robust effect at 10 ng/ml (0.6 nM), thus at a 10-fold molar excess, while complete inhibition was observed at 100 ng/ml (5.6 nM), corresponding to a 100-fold molar excess. These experiments further confirm that our in-house production platform allows for successful production and purification of biologically active recombinant cytokines.

In contrast, none of the IL-38 proteins tested had a major effect on IL-1α–induced IL-8 production at concentrations corresponding to a 10 to 1000-fold molar excess as compared to IL-1α. Only the aa20-152 IL-38:Fc-KIH fusion protein reduced IL-8 production by approximately 25% at the highest concentration tested (3000 ng/ml, corresponding to 38.5 nM), representing a 700-fold molar excess over IL-1α. As expected, IL-36Ra (Fig. 4A), as well as unfused Fc-KIH IgG1 (Figure S3C), used as negative controls, had no effect on this IL-1R1-mediated response. In parallel, we assessed IL-6 production in NHK cells in the same experimental conditions (Figure S5C). As for IL-8 production, sIL-1Ra and icIL-1Ra1 completely inhibited the effect of IL-1α at a 10-100-fold molar excess, while neither the IL-38 proteins nor

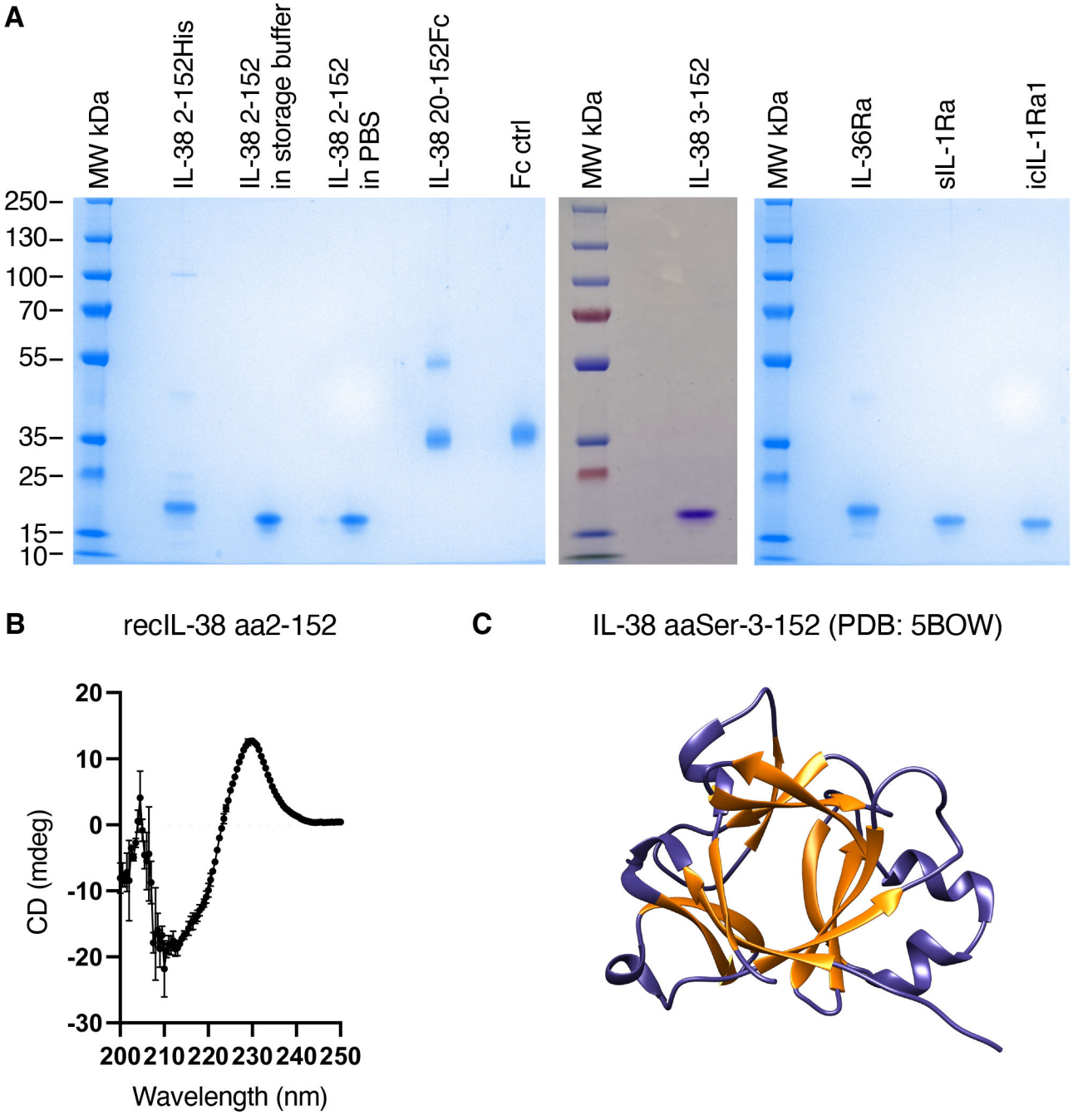


Fig. 2 Purity of recombinant cytokine preparations and folding of recombinant aa2-152 IL-38. **A**. To assess the purity of the recombinant protein preparations used in this study, 1 µg of each sample (aa2-152 IL-38 with C-terminal His tag, aa2-152 IL-38 in storage buffer and in PBS, aa20-152 IL-38:Fc-KIH fusion protein, Fc-KIH IgG1 control, left gel; aa3-152 IL-38, middle gel; IL-36Ra, sIL-1Ra, icIL-1Ra1, right gel) were fractionated by SDS-PAGE and stained with Coomassie Blue. Based on calculated molecular weights (MW), expected sizes are: 18 kDa for aa2-152His IL-38 and icIL-1Ra1, 17 kDa for aa2-152 IL-38, aa3-152 IL-38, IL-36Ra and sIL-1Ra1, 28 kDa and 50 kDa for IL-38:Fc-KIH, 28 kDa for Fc-KIH IgG1 control. The size (kDa) of MW markers is indicated on the left. Storage buffer: 30 mM Hepes pH 7, 500mM NaCl, 5% glycerol, 1 mM β-ME. Fc ctrl: Fc-KIH IgG1. **B**. Folding of recombinant IL-38 aa2-152 was analyzed by circular dichroism. The observed percentage of β-strands was approximately 40%. CD, circular dichroism; Mdeg, measured ellipticity. **C**. X-ray diffraction crystal structure of the human IL-38 protein without the initiator methionine and with a Cys-to-Ser mutation at position 2 (PDB: 5BOW). β-strands (orange) represent 40.1% of the sequence, while the remaining regions (dark blue) are structured as coils and short α-helices

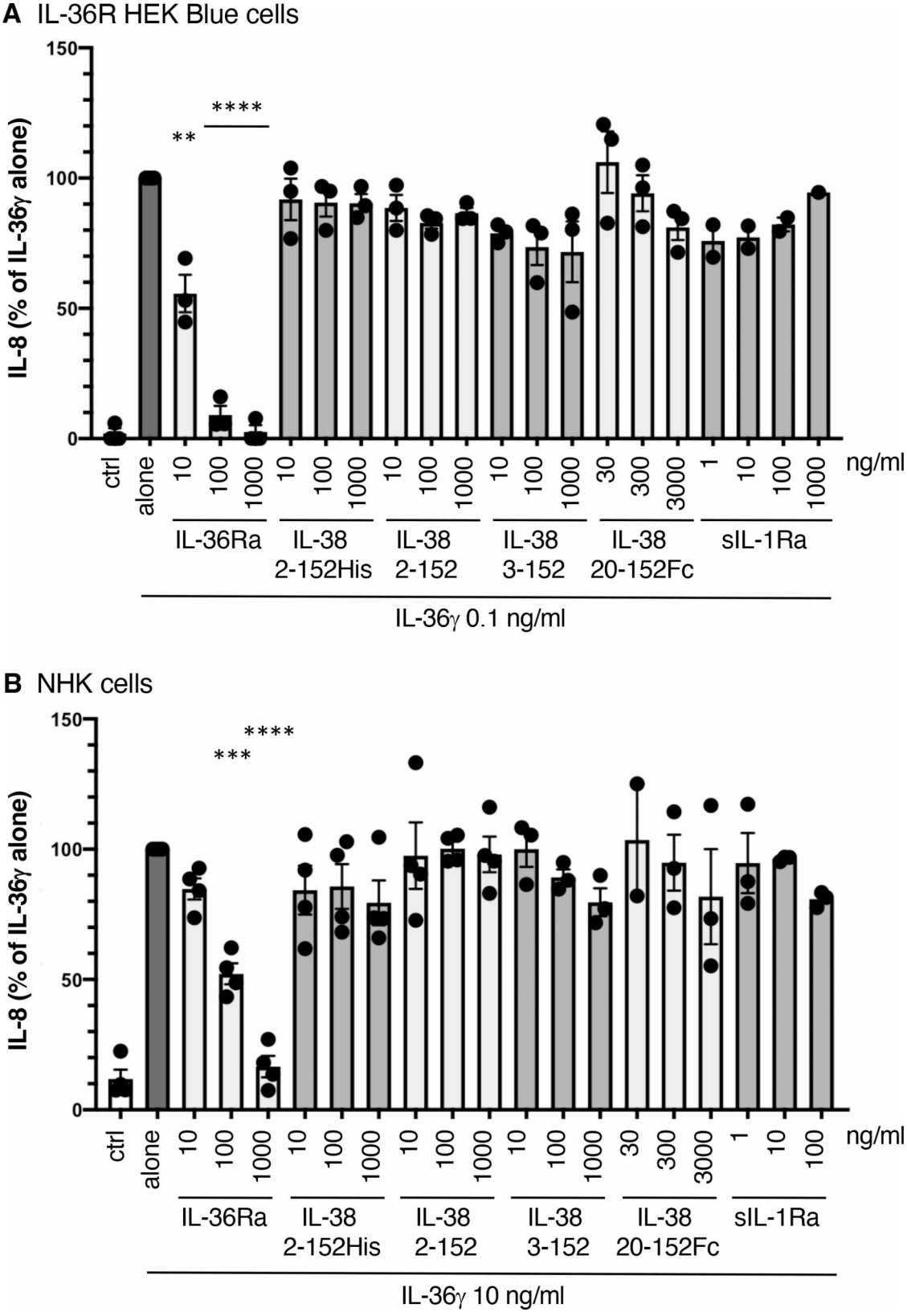


Fig. 3 (See legend on next page.)

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Fig. 3 Recombinant IL-38 proteins do not affect IL-36γ-induced IL-8 production in IL-36R HEK Blue and NHK cells. **A**. IL-36R HEK Blue and **B**. NHK cells were preincubated with recombinant human IL-38 variants (aa2-152 with C-terminal His tag; aa2-152; aa3-152; aa20-152 IL-38:Fc-KIH fusion protein) at indicated concentrations (10-3000 ng/ml, corresponding to 0.4–58.8 nM; see Table 1), before stimulation with IL-36γ (0.1 ng/ml or 10 ng/ml, respectively, corresponding to 0.006 or 0.6 nM) for 24 h. IL-8 production was assessed by ELISA in culture supernatants. Recombinant human IL-36Ra (10-1000 ng/ml, corresponding to 0.6–58.8 nM) was used as a positive control to inhibit IL-36γ. Recombinant human sIL-1Ra (1-1000 ng/ml, corresponding to 0.06–58.8 nM) was used as a negative control. Results are expressed in % of the IL-8 production observed with IL-36γ alone. Each dot represents the mean of 3 technical replicates in an independent experiment. Results are shown as individual values and mean ± SEM for 3 (**A**) or 4 (**B**) independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. IL-36γ alone, as assessed by ANOVA, followed by Dunnett's multiple comparisons test

IL-36Ra had any effect. In contrast to IL-8 production, IL-6 secretion was not affected by aa20-152 IL-38:Fc-KIH, even at the highest concentration used.

Finally, we analyzed the effects of the different recombinant IL-38 proteins on IL-1α–induced IL-8 production the IL-36R HEK Blue reporter cell line. Although these cells are not specifically engineered to respond strongly to IL-1, they endogenously express IL-1R1 (Figure S5B), which allows for induction of IL-8 production by IL-1α (Fig. 4B). In IL-36R HEK Blue cells, the effect of 1 ng/ml (0.06 nM) IL-1α on IL-8 secretion was partially blocked by 1 ng/ml (0.06 nM) sIL-1Ra or icIL-1Ra1, thus at a 1:1 molar ratio, and completely inhibited at 10 ng/ml (0.6 nM) sIL-1Ra and icIL-1Ra1, thus at a 10-fold molar excess (Fig. 4B). In contrast, none of the IL-38 proteins tested had any major effect on IL-1α–induced IL-8 production. Again, only the aa20-152 IL-38:Fc-KIH fusion protein reduced IL-8 production by approximately 30% at the highest concentration tested, corresponding to a 700-fold molar excess over IL-1α. Finally, as expected, IL-36Ra (Fig. 4B) and Fc-KIH IgG1 (Figure S3D) had no effect on this IL-1R-dependent readout. Taken together, this series of experiments indicates that, in contrast to sIL-1Ra and icIL-1Ra1, various IL-38 variants tested do not efficiently antagonize IL-1α activity in two different IL-1R1 expressing cell types.

Discussion

In this study, we described the native N-terminus of the IL-38 protein produced by human keratinocytes, which starts at cysteine 2. We then evaluated the effects of aa2-152 IL-38 and of two shorter forms of the protein on IL-36R- and IL-1R1-mediated responses in two different cell lines on different readouts. We did not obtain evidence for efficient IL-36R or IL-1R1 antagonist activity of any of the recombinant IL-38 variants tested. This was paralleled by only moderate affinity of the tested IL-38 proteins for IL-36R in a direct binding assay.

MS/MS analysis of the IL-38 protein produced at steady state in the overexpressing NHK/38 keratinocyte cell line, as well as of endogenous IL-38 isolated from healthy human epidermis revealed the presence of an acetylated N-terminal peptide starting at cysteine 2, in agreement with previous observations in A549 lung carcinoma cells [9]. N-terminal methionine excision and N-terminal acetylation are two commonly observed protein modifications in eukaryotic cells [36, 37] and these co-translational processes likely account for the generation of aa2-152 IL-38, as the naturally occurring 'full length' form of this protein. Indeed, methionyl aminopeptidases typically remove the initiator methionine from proteins, in which it is followed by a small residue, such as the cysteine at position 2 in IL-38 [36]. In our experimental conditions, we did not obtain any evidence for the existence of shorter N-terminally truncated forms, such as the aa3-152, aa7-172 or aa20-152 IL-38 variants previously proposed to possess more robust anti-inflammatory activity than full length IL-38 [9, 17–21]. This does of course not exclude the possibility that additional truncations may occur, for example, during inflammation or cell death.

We therefore compared the effects of aa2-152, as well as two shorter forms of recombinant IL-38 on IL-36R- and IL-1R1-mediated responses in two different cell lines. The sensitivity of IL-36R overexpressing HEK Blue cells to IL-36 stimulation was higher than that of NHK cells, correlating with higher cell surface IL-36R expression. Consequently, a higher IL-36Ra/IL-36γ ratio was required to efficiently block IL-36R-mediated responses in IL-36R HEK Blue than in NHK cells, presumably because more antagonist was needed to achieve

Table 2 Recombinant IL-38 proteins bind to the IL-36R with moderate affinity

recombinant protein	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/s)	kd2 (1/s)	KD (M)
IL-36Ra	1.29E + 04	2.71E-02	2.82E-03	6.61E-05	4.82E-08
IL-38 2-152His	7.17E + 03	1.48E-02	3.58E-03	6.71E-04	3.25E-07
IL-38 2-152	9.67E + 03	2.26E-02	3.92E-03	7.25E-04	3.66E-07
IL-38 3-152	2.81E + 03	1.63E-02	4.16E-03	9.47E-04	1.08E-06
icIL-1Ra1	9.63E + 03	3.19E-01	5.25E-05	5.38E-02	3.31E-05

Binding of recombinant human IL-36Ra, IL-38 variants (aa 2-152 with C-terminal His tag; aa 2-152; aa3-152) and icIL-1Ra1 to an immobilized IL-36R-Fc chimeric protein was analyzed by SPR using a two-state reaction model. The binding results are reported as association (ka) and dissociation (kd) rate constants and binding affinity (KD)

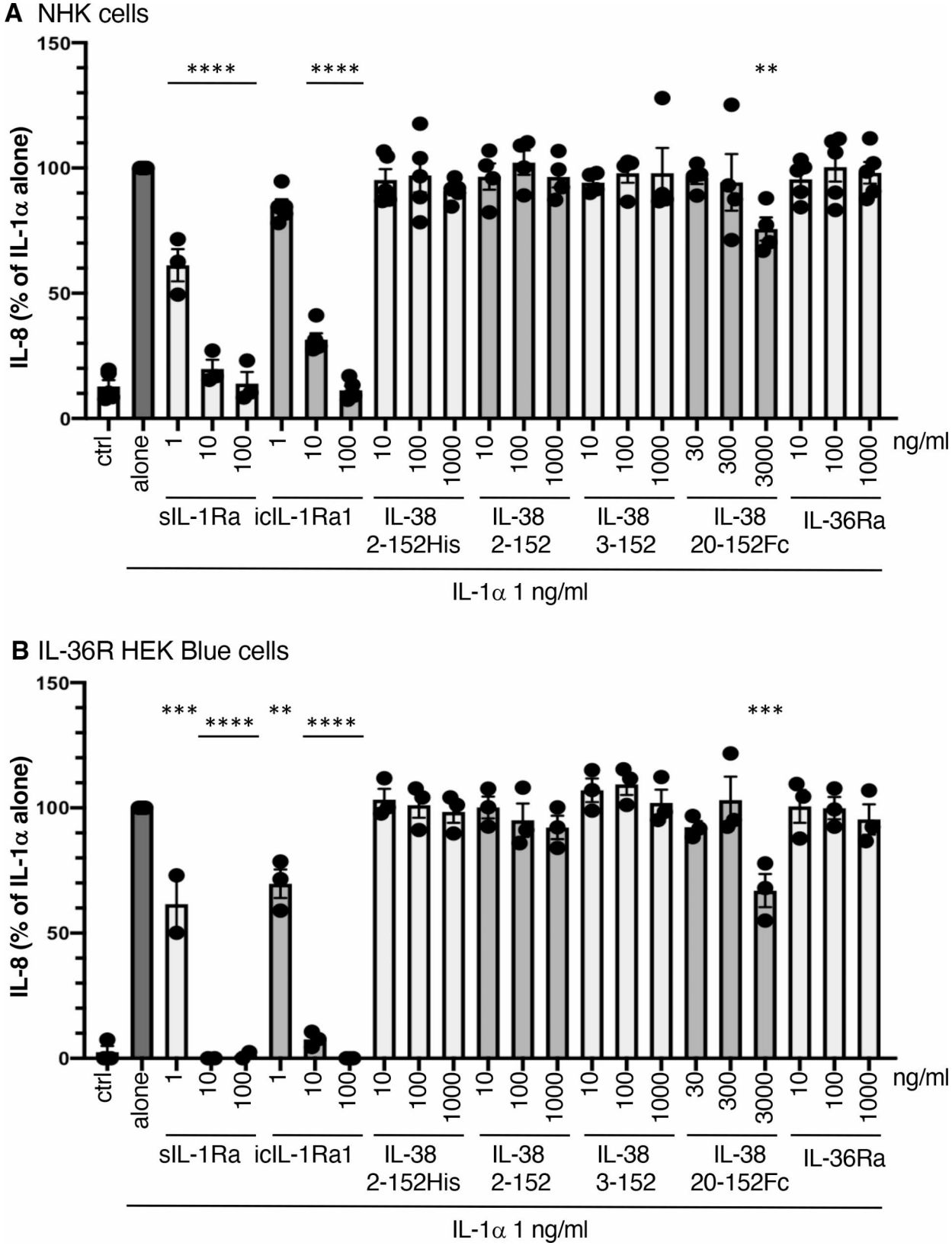

Fig. 4 (See legend on next page.)

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Fig. 4 Recombinant IL-38 proteins do not globally affect IL-1α-induced IL-8 production in IL-36R HEK Blue and NHK cells. **A**. NHK and **B**. IL-36R HEK Blue cells were preincubated with recombinant human IL-38 variants (aa2-152 with C-terminal His tag; aa2-152; aa3-152; aa20-152 IL-38:Fc-KIH fusion protein) at indicated concentrations (10-3000 ng/ml, corresponding to 0.4–58.8 nM; see Table 1), before stimulation with IL-1α (1 ng/ml, corresponding to 0.06 nM) for 24 h. IL-8 production was assessed by ELISA in culture supernatants. Recombinant human sIL-1Ra (1-100 ng/ml, corresponding to 0.06–5.9 nM) and recombinant icIL-1Ra1 (1-100 ng/ml, corresponding to 0.06–5.6 nM) were used as positive controls to inhibit IL-1α. Recombinant human IL-36Ra (10-1000 ng/ml, corresponding to 0.6–58.8 nM) was used as a negative control. Results are expressed in % of the IL-8 production observed with IL-1α alone. Each dot represents the mean of 3 technical replicates in an independent experiment. Results are shown as individual values and mean ± SEM for 5 (**A**) or 3 (**B**) independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. IL-1α alone, as assessed by ANOVA, followed by Dunnett's multiple comparisons test

complete receptor occupancy. The different IL-38 variants were used in the same dose range as IL-36Ra, but did not exert any significant effect on IL-36R-mediated responses, even in NHK cells where a lower antagonist/agonist ratio was required for inhibition of IL-36γ by IL-36Ra. Our SPR data showing that IL-38 binds to the IL-36R with lower affinity than IL-36Ra might provide a mechanistic explanation for this observation. Similarly, in contrast to sIL-1Ra and icIL-1Ra1 used in the same dose range, the different IL-38 proteins also failed to efficiently antagonize IL-1α activity in two different IL-1R1 expressing cell types. In fact, when assayed for either IL-36R or IL-1R1 antagonist activity, the tested IL-38 variants globally behaved like the cytokines that we included as negative controls (IL-1Ra for IL-36R and IL-36Ra for IL-1R1) because they were known to target a different receptor. Collectively, these observations indicate that several recombinant forms of IL-38 obtained from different sources, both commercial and produced in-house, fail to efficiently antagonize IL-36R or IL-1R1 activity. However, we would like to emphasize that these conclusions relate specifically to the proteins and conditions that we tested, and should not be extrapolated to other experimental systems or to reported activities of IL-38 that are independent of the main research question addressed in this study.

CD analysis confirmed a high β-strand content of in-house produced aa2-152 IL-38, consistent with correct folding of its β-trefoil structure. The aa3-152 IL-38 variant was produced in identical conditions and removal of a single additional N-terminal amino acid is not expected to impair the folding of the protein. Indeed, in other family members, small N-terminal truncations are rather known to enhance bioactivity [3]. Furthermore, icIL-1Ra1, which was also produced in parallel, was functional, with a bioactivity similar to that of commercial sIL-1Ra, again confirming correct folding. Since we did not generate CD spectra for the commercial recombinant IL-38 proteins used in this study, we can not formally conclude on folding status of these variants.

Full-length and N-terminally truncated recombinant forms of IL-38 were similarly inefficient in our study. Proteolytic activation of pro-inflammatory IL-1 family cytokines by immune cell- or pathogen-derived proteases has emerged as an important mechanism involved in danger sensing [38]. To our knowledge, there is however no evidence showing that the activity of the two anti-inflammatory family members IL-1Ra and IL-36Ra is similarly enhanced by post-translational N-terminal processing. Like IL-38, IL-36Ra was shown to undergo co-translational removal of its initiator methionine, which is essential for its activity [3, 39], but we are not aware of any description of further N-terminal processing of this protein by other proteases. IL-1Ra exists in different isoforms, which differ at their N-terminus [35]. The icIL-1Ra1 isoform has 7 additional N-terminal aa as compared to sIL-1Ra [22]. Yet, as confirmed also in the present study, the two isoforms have similar biological activity [40], indicating that its exact N-terminal sequence is not an essential determinant of IL-1Ra function. The assumption that the anti-inflammatory IL-38 activity needs to be enhanced by N-terminal processing is thus not supported by analogy to the two IL-1 family receptor antagonists.

Based on our data, we can not exclude that different preparations, or yet to be described forms of IL-38 might possess more significant IL-36R or IL-1R1 antagonist activity. Nevertheless, we believe that our conclusions may apply to IL-38 in general. Indeed, we complemented the present work with in vitro and in vivo studies, in which we overexpressed the IL-38 protein in keratinocytes, on the premise that, since keratinocytes are natural IL-38 producing cells, they can be expected to produce a functional form of the protein [41, 42]. In vivo, we observed selective anti-inflammatory effects of IL-38, as well as reduced desquamation, in a mouse model of psoriasis, demonstrating the bioactivity of the overexpressed protein [42]. Interestingly however, excessive IL-38 signaling did not phenocopy IL-36R or IL-1R1 deficiency, which more broadly affect the IL-17 axis in this model [43, 44], indicating that IL-38 was not acting as an IL-36R or IL-1R1 antagonist. Similarly, in vitro, IL-38 overexpression in keratinocytes did not affect IL-36 or IL-1-induced inflammatory responses, while icIL-1Ra1 overexpression efficiently blocked the effects of IL-1α [41].

Finally, we observed a marginal effect of a 700-fold molar excess of aa20-152 IL-38:Fc-KIH specifically on one of the two tested IL-1α-induced readouts. Given its selectivity and the extremely high dose of aa20-152 IL-38:Fc-KIH required, this effect seems unlikely to reflect a *bona fide* receptor antagonist activity of IL-38 competitively inhibiting agonist-induced IL-1R1 activation. In

fact, the 19 aa N-terminal truncation in this IL-38 variant deletes the entire first β-sheet and part of the second β-sheet of the protein's characteristic 12 β-sheet trefoil fold and was predicted to destabilize this structure, which is essential for receptor binding and activity of other IL-1 family cytokines [30]. In addition, IL-1Ra completely inhibits IL-1α activity at a 100-fold lower concentration. It is thus difficult to imagine that this particular truncated form of IL-38 might contribute significantly to the regulation of IL-1 activity in physiological situations in vivo, where even the smallest amounts of IL-1Ra would make its marginal activity redundant.

Conclusions

Our results did not provide evidence for consistent and robust inhibitory effects of any of the recombinant IL-38 variants tested on IL-36R or IL-1R1-mediated responses. The mechanisms underlying reported anti-inflammatory effects of IL-38 are thus still unclear, but seem unlikely to be related to classical IL-36R or IL-1R1 antagonism.

Abbreviations

Aa	Amino acid
HEK	Human embryonic kidney
icIL-1Ra	Intracellular interleukin-1 receptor antagonist
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAPL1	Interleukin-1 receptor accessory protein-like 1
IL-36R	Interleukin-36 receptor
IL-36Ra	Interleukin-36 receptor antagonist
KIH	Knobs-in-holes
LC-ESI-MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
NHK	Normal human keratinocyte
PSM	Peptide spectrum matches
SEC	Size exchange chromatography
SEAP	Secreted embryonic alkaline phosphatase
sIL-1Ra	Secreted interleukin-1 receptor antagonist
SPR	Surface plasmon resonance

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-025-02035-z.

Supplementary Material 1: **Figure S1.** Preparative immunoprecipitation of the IL-38 protein from NHK/38 cells for LC-ESI-MS/MS analysis. IL-38 was immunoprecipitated from NHK/38 cell lysates as in Fig. 1A. Input lysates (NHK/38 lysate) and immunoprecipitated proteins (IP anti-IL-38) were run in triplicate by SDS-PAGE. IL-38 was detected by Western blotting (left), and total proteins were stained with silver stain (middle) or Coomassie Blue (right). Bands migrating at the expected size for IL-38 (17 kD) are highlighted on the silver and Coomassie Blue stained gels (white dashed boxes). The boxed band was excised from the Coomassie Blue stained gel and the proteins contained therein were reduced, alkylated and in-gel digested for LC-ESI-MS/MS. The size (kDa) of MW markers is indicated on the right. The results are representative of 2 independent experiments.

Supplementary Material 2: **Figure S2.** Characterization of the IL-36γ response in IL-36R HEK Blue and NHK cells. A. IL-36R HEK Blue cells were stimulated with IL-36γ (0.001-0.1 ng/ml, corresponding to 0.06 pM-0.006 nM) for 24 h. SEAP production was assessed in culture supernatants. B. NHK cells were stimulated with IL-36γ (1–50 ng/ml, corresponding to 0.06–2.9 nM) for 24 h. IL-8 production was assessed by ELISA in culture supernatants. A-B. Results are shown as mean ± SEM for $n = 3$ technical replicates in a representative experiment. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. control, as assessed by ANOVA, followed by Dunnett's multiple comparisons test. C. Cell surface IL-36R expression levels were examined by flow cytometry. A representative normalized histogram overlay (left panel) and mean fluorescence intensity (MFI) quantification (right panel) are shown for parental HEK, IL-36R HEK Blue and NHK cells. MFI is represented as individual values and mean ± SEM for 3 independent experiments. ***$p < 0.001$ NHK vs. IL-36R HEK Blue cells, ****$p < 0.0001$ IL-36R HEK Blue vs. parental HEK cells, as assessed by ANOVA followed by Sidak's multiple comparisons test. D. NHK cells were preincubated with recombinant human IL-38 variants (aa2-152 with C-terminal His tag; aa2-152; aa3-152; aa20-152 IL-38:Fc-KIH fusion protein) at indicated concentrations (10-3000 ng/ml, corresponding to 0.4–58.8 nM; see Table 1) before stimulation with IL-36γ (10 ng/ml, corresponding to 0.6 nM) for 24 h. IL-6 production was assessed by ELISA in culture supernatants. Recombinant human IL-36Ra (10-1000 ng/ml, corresponding to 0.6–58.8 nM) was used as a positive control to inhibit IL-36γ. Recombinant human sIL-1Ra (1-100 ng/ml, corresponding to 0.06–5.9 nM) was used as a negative control. Results are expressed in % of the IL-6 production observed with IL-36γ alone. Each dot represents the mean of 3 technical replicates in an independent experiment. Results are shown as individual values and mean ± SEM for 4 independent experiments. **$p < 0.01$, ****$p < 0.0001$ vs. IL-36γ alone, as assessed by ANOVA, followed by Dunnett's multiple comparisons test.

Supplementary Material 3: **Figure S3.** Effect of unfused Fc-KIH control IgG1 on IL-36γ- or IL-1α-induced IL-8 production in IL-36R HEK Blue and NHK cells. A. IL-36R HEK Blue and B. NHK cells were preincubated with recombinant human aa20-152 IL-38:Fc-KIH fusion protein (IL-38 20-152Fc) or with an Fc-KIH IgG1 negative control (Fc ctrl) at indicated concentrations (17-3000 ng/ml, corresponding to 0.4–60.7 nM; see Table 1) before stimulation with IL-36γ (0.1 ng/ml or 10 ng/ml, respectively, corresponding to 0.006 or 0.6 nM) for 24 h. IL-8 production was assessed by ELISA in culture supernatants. Results are expressed in % of the IL-8 production observed with IL-36γ alone. Each dot represents the mean of 3 technical replicates in an independent experiment. Results are shown as individual values and mean ± SEM for 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. IL-36γ alone, as assessed by ANOVA, followed by Dunnett's multiple comparisons test. C. NHK and D. IL-36R HEK Blue cells were preincubated with recombinant human aa20-152 IL-38:Fc-KIH fusion protein (IL-38 20-152Fc) or with an Fc-KIH IgG1 negative control (Fc ctrl) at indicated concentrations (17-3000 ng/ml, corresponding to 0.4–60.7 nM; see Table 1) before stimulation with IL-1α (1 ng/ml, corresponding to 0.06 nM) for 24 h. IL-8 production was assessed by ELISA in culture supernatants. Results are expressed in % of the IL-8 production observed with IL-1α alone. Each dot represents the mean of 3 technical replicates in an independent experiment. Results are shown as individual values and mean ± SEM for 3 (NHK) or 2 (IL-36R HEK Blue) independent experiments. *$p < 0.05$, ****$p < 0.0001$ vs. IL-1α alone, as assessed by ANOVA, followed by Dunnett's multiple comparisons test.

Supplementary Material 4: **Figure S4.** Recombinant IL-38 proteins alone do not induce SEAP or IL-8 production in IL-36R HEK Blue and NHK cells. A. IL-36R HEK Blue and B. NHK cells were incubated with recombinant human IL-36Ra, IL-38 variants (aa2-152 with C-terminal His tag; aa2-152; aa3-152; aa20-152 IL-38:Fc-KIH fusion protein), sIL-1Ra, or icIL-1Ra1 at indicated concentrations (1-3000 ng/ml, corresponding to 0.06–58.8 nM; see Table 1) or stimulated with IL-36γ (0.1 ng/ml or 10 ng/ml, respectively, corresponding to 0.006 or 0.6 nM) or IL-1α (1 ng/ml, corresponding to 0.06 nM) for 24 h before SEAP or IL-8 production was assessed in culture supernatants. Results are shown as mean ± SEM for $n = 3$ technical replicates in a representative experiment out of $n = 2$ (aa2-152 IL-38), 5 (icIL-1Ra1), 6 (aa2-152His IL-38), 7 (3-152 IL-38, aa20-152 IL-38:Fc-KIH, sIL-1Ra), or 8 (IL-36Ra). ****$p < 0.0001$ vs. control, as assessed by ANOVA, followed by Dunnett's multiple comparisons test.

Supplementary Material 5: **Figure S5.** Characterization of the IL-1α response in NHK and IL-36R HEK Blue cells. A. NHK cells were stimulated with IL-1α (0.1 and 1 ng/ml, corresponding to 0.006 and 0.06 nM) for 24 h. IL-8 production was assessed by ELISA in culture supernatants. Results are shown as mean ± SEM for $n = 3$ technical replicates in a representative experiment. **p < 0.01, ****p < 0.0001 vs. control, as assessed by

ANOVA, followed by Dunnett's multiple comparisons test. B. Cell surface IL-1R1 expression levels were examined by flow cytometry. A representative normalized histogram overlay (left panel) and mean fluorescence intensity (MFI) quantification (right panel) are shown for parental HEK, IL-36R HEK Blue and NHK cells. MFI is represented as individual values and mean ± SEM for 2 independent experiments. C. NHK cells were preincubated with recombinant human IL-38 variants (aa 2-152 with C-terminal His tag; aa 2-152; aa3-152; aa20-152 IL-38:Fc-KIH fusion protein) at indicated concentrations (10-3000 ng/ml, corresponding to 0.4–58.8 nM; see Table 1) before stimulation with IL-1α (1 ng/ml, corresponding to 0.06 nM) for 24 h. IL-6 production was assessed by ELISA in culture supernatants. Recombinant human sIL-1Ra (1-100 ng/ml, corresponding to 0.06–5.9 nM) and recombinant icIL-1Ra1 (1-100 ng/ml, corresponding to 0.06–5.6 nM) were used as positive controls to inhibit IL-1α. Recombinant human IL-36Ra (10-1000 ng/ml, corresponding to 0.6–58.8 nM) was used as a negative control. Results are expressed in % of the IL-6 production observed with IL-1α alone. Each dot represents the mean of 3 technical replicates in an independent experiment. Results are shown as individual values and mean ± SEM for 4 independent experiments. ****$p < 0.0001$ vs. IL-1α alone, as assessed by ANOVA, followed by Dunnett's multiple comparisons test.

Supplementary Material 6

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Author contributions
ADB: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing; DTA: Methodology, Investigation, Formal analysis; AH: Methodology, Investigation, Formal analysis; GC: Methodology, Investigation, Formal analysis; JT: Methodology, Investigation; MM: Methodology, Investigation, Formal analysis; AFM: Conceptualization, Writing – review & editing; EM: Methodology, Investigation, Formal analysis, Writing – review & editing; GP: Conceptualization, Methodology, Formal analysis, Project administration, Visualization, Writing – original draft, Funding acquisition, Supervision. All authors read and approved the final manuscript.

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Data availability
The datasets generated and analyzed during the current study are available in the Yareta repository, https://doi.org/10.26037/yareta:btrvdygarvahzcyvhq6h7e56me.

Declarations

Ethics approval and consent to participate
Skin biopsies were taken from healthy adults undergoing surgery at the Department of Plastic and Reconstructive Surgery of the Geneva University Hospitals in Switzerland. This study was conducted according to the Declaration of Helsinki, and approved by the local ethics committee of the University Hospitals of Geneva (protocol number 2017 – 00700). Written informed consent was obtained from each individual.

Consent for publication
not applicable.

Competing interests
The authors declare no competing interests.

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